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# GROWTH

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# OOGENESIS IN ADULT *DROSOPHILA MELANOGASTER*. III. RADIATION-INDUCED OVARIAN TUMORS[1]

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(Received June 27, 1957)

## INTRODUCTION

Recently studies (King, Rubinson and Smith, 1956; King, Darrow and Kaye, 1956; King, 1957) have been published which describe both normal oogenesis in the fruit fly, *Drosophila melanogaster*, and also the relation between sensitivity to radiation-induced mutation and the stage in development of the germ cells of females. While these studies were in progress ovarian tumors were discovered in both the treated and control material. It is the purpose of this paper to describe further studies of these ovarian tumors and to discuss their possible mode of origin. At this early stage in our knowledge concerning the ovarian tumors of *Drosophila* it is perhaps wise to suspend judgment as to their relationship with mammalian tumors.

## MATERIAL AND METHODS

Adult *Drosophila melanogaster* belonging to the Oregon-R, wild type strain were used in this study. All flies were raised at 25°C. Female flies were irradiated with 4,000r of Cobalt$^{60}$ gamma rays ($\sim$ 100 r/second) when they were less than 1 hour old. They were then placed with untreated males on "enriched" medium (King and Wood, 1955) for 12 days. They were killed subsequently, and Feulgen-stained whole mounts were made of their ovaries (Cf. King, Rubinson, and Smith, 1956, for the procedures followed).

## RESULTS AND CONCLUSIONS

*Tumor incidence:*

Approximately 67,000 developing eggs were observed. The results are summarized in Table 1. The treatment has increased the tumor

[1] Research supported by the U.S. Atomic Energy Commission (contract No. AT (11-1)-89, Project 12) and the Graduate School of Northwestern University.

TABLE 1
Ovarian Tumors in Control and Treated Material

| | *Control* | | | | *Treated* |
|---|---|---|---|---|---|
| Total developing eggs | 39,900 | | | | 26,880 |
| Total ovarioles | 5,700 | | | | 4,480 |
| Total ovaries | 475 | | | | 448 |
| Total tumors | 2 | | | | 35 |
| Tumors/10,000 developing eggs | 0.5 | | | | 13.0 |
| | *Tumor Distribution* | | | | |
| Number of tumors per ovariole | 1 | 2 | 3 | 4 | 5 |
| Number of cases | 9 | 5 | 3 | 1 | 1 |

frequency by 26 times. Furthermore, whenever two or more tumors occur in the same ovariole the tumors are adjacent to one another (cf. Figure 1). This finding argues for a clonal origin of the tumors. If this is the situation, it follows that 18 tumor-inducing events occurred in the treated series; one in the control. Cases were observed where a tumorous chamber was preceded or followed by an apparently normal chamber.

*Tumor cytology:*

A cytological study using phase and bright field microscopy was made of the 37 recorded tumors. Generally the tumorous follicles are packed with cells which have an average diameter of 7 micra (Cf. Figures 50 and 51 (King, Rubinson and Smith, 1956)). The nuclei of some of these tumor cells contain a characteristic karyosome and are therefore morphologically similar to oocytes. Twenty-seven tumors showed such oocyte-like cells. These karyosome-containing cells are distributed randomly throughout the tumor. Their number is always greater than one but less than 1/16th of the total cell number. The remaining tumor cells are characterized by pale-staining interphase nuclei. Thirty tumors showed such cells. Dividing cells were observed in 35 out of the 37 tumors.

In four tumors a few cells were seen which appear to have differentiated into cells resembling stage 3 nurse cells. In one instance a tumor was observed which resembled a stage 8 oocyte both in over-all size and in the morphology of the follicular epithelium. Generally the follicular epithelium surrounding the tumor is composed of cuboidal cells. In this case, however, the posterior epithelium was columnar and the anterior epithelium squamous. Furthermore, the nuclei of the

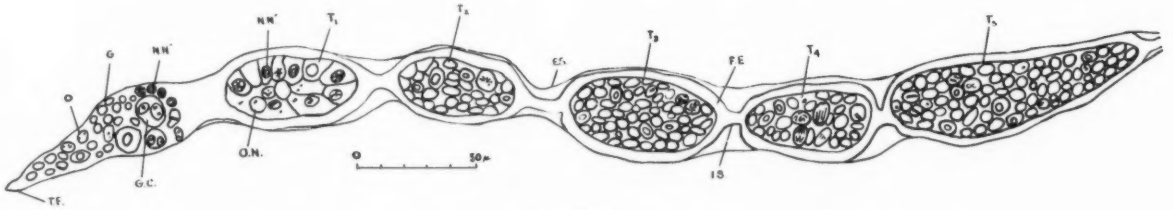


FIGURE 1

A camera lucida outline drawing of an ovariole from an irradiated ovary showing five tumors adjacent to one another. E.S., epithelial sheath; F.E., follicular epithelium; G, germarium; G.C., germarial cyst; I.S., interfollicular stalk, N.N'., nurse-like nucleus; O, cogonium; T, tumor; T.F., terminal filament.

tumor cells were almost competely devoid of Feulgen-positive material. One other tumor composed of Feulgen-negative cells was observed. Finally, an exceptional tumor occurred containing cavities which held blobs of Feulgen-positive material. These blobs resemble degenerating nurse cell nuclei.

*Distribution of tumors with respect to size:*

The tumors observed were spherical or spheroidal. They were measured using a calibrated ocular micrometer, and their volumes were subsequently calculated. The diameter of the average cell comprising the tumor was also determined. In the case of tumors shaped like prolate spheroids the diameter of a sphere of equal volume was calculated. The minimum number, N, of spherical cells of diameter $d_1$, enclosed in the spherical tumor of diameter $d_2$ is given by the equation $N = 0.7404(d_2/d_1)^3$. The tumor cells vary in diameter between 6 and 10 micra. The tumors vary in size from a stage 2 to a stage 9 chamber. The smallest and largest tumors differ in volume by a factor of 200x. Table 2 shows a plot of the distribution of tumors of various sizes. The original cells of 70% of all tumors have undergone an average of 2 to 6 non-synchronous, supernumerary divisions during the 12-day interval that followed irradiation.

TABLE 2
The Distribution of Tumors of Various Sizes

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | | 240 | | | | | | |
| | | | 210 | | | | | | |
| | | | 210 | 460 | | | | | |
| | | | 200 | 440 | | | | | |
| | | 120 | 190 | 380 | 940 | | | | |
| | | 90 | 150 | 360 | 820 | | | | |
| 30 | | 90 | 140 | 330 | 740 | | | | |
| 30 | 60 | 80 | 140 | 320 | 570 | 1750 | 3380 | | |
| 23 | 60 | 70 | 130 | 270 | 540 | 1580 | 2500 | 4530 | 8700 |
| 0–1 | 1–2 | 2–3 | 3–4 | 4–5 | 5–6 | 6–7 | 7–8 | 8–9 | 9–10 |

The numbers above the horizontal lines give the calculated minimum number of cells per tumor. The numbers below the horizontal lines give the minimum average number of supernumerary divisions which occurred to each of the sixteen original cells of the chamber. Thus, in nine tumors each of the original cells have undergone 3 or 4 supernumerary divisions.

## Discussion

*The normal origin of the developing egg:*

The two ovaries of the fruit fly are each subdivided into 12 egg tubes or ovarioles. The ovariole is differentiated into an anterior germarium and a series of egg chambers. The germarium can be divided into a series of four regions (1 being the most anterior). In region 1 are found mitotically active oogonia. The reproductive system contains roughly 1000 such cells, and each one must divide at least twice during the lifetime of the insect, since the fly under optimal conditions produces over 3000 eggs. The duplication of genetic material and subsequent cell division appear to go on continually in region 1 (Cf. Table 3). On the other hand in region 2 cell division is limited. A cell entering region 2 from region 1 can undergo four consecutive, synchronous divisions, and then cytokinesis ceases. The sixteen cells so produced

TABLE 3
The Zonation of the *Drosophila* germarium

| Region | Criterion | Length (micra) | Continued synthesis of genetic material | Continued cytokinesis |
|---|---|---|---|---|
| 1 | Zone of unlimited division of oogonia | 20 | + | + |
| 2 | Zone of limited division of cyst cells | 15 | + | limited |
| 3 | Zone of determination of nurse cells | 20 | + | — |
| 4 | Zone of determination of the oocyte | 5 | — | — |

enter regions 3 and 4, and they form a spherical cyst which eventually is surrounded by a follicular epithelium. The most posterior cell of the cyst always differentiates into the oocyte (the others into nurse cells), and this is the reason for initially postulating regions 3 and 4. The nurse cells which are determined in region 3 never divide but continue to synthesize genetic material, and as a consequence the DNA content per nucleus increases markedly (Schultz, 1956). The oocyte which is determined in region 4 contains 4 times the haploid amount of DNA throughout development.

A model which allows this complex sequence of events to be given

a superficially simple explanation provides that the germarium is differentiated into a series of three or four biochemically distinct cytoplasmic zones. As a cell enters first one and then another zone, first one and then another portion of the cell's genome is affected in a characteristic fashion which in turn alters the subsequent cytological behavior of the cell. Thus, the inhibitory substance I contained in region 2 (but not 1) interacts with the genome in some unknown but precise fashion to bring cell division to an irreversible halt; but only after four consecutive divisions have occurred and a 16-cell aggregate exists. I does not interfere with DNA synthesis; this is halted by a different substance produced in region 4 (but not in 3).

*The origin of tumors:*

The model allows us to postulate a mechanism of tumor formation. It is simply assumed that radiation inactivates that portion of the genome of an oogonial cell which interacts with substance I when it is present in region 2. This mutation is passed on to the progeny of the oogonial cell. Let us assume that one such mutated cell divides until it produces 16 cells, and a follicle layer of non-mutant cells encircles them. The follicle so produced would form the first chamber in the ovariole, but the germ cells within it continue to divide. A second mutated sister oogonial cell might next produce another germarial cyst in the same fashion and this eventually would supplant the first chamber. Now the first and second chambers would be tumorous. Next a non-mutant oogonium might produce a germarial cyst. The result of this postulated sequence of events would be an ovariole with three chambers: the anterior one normal, and the second and third tumorous. If the rate of cell division often exceeded the rate of DNA replication, the resulting cells would eventually come to contain little Feulgen-positive material in their nuclei and this situation has been observed. In rare instances the reverse might occur, and as a result cells with nuclei reminiscent of nurse cells would be produced. Since the check on mitosis is eliminated new cells might be pushed continually into the region where for a certain limited period of time determination of the oocyte occurs. These cells would be transformed to karyosome-containing cells and would lose the power of further DNA synthesis. They in turn might be displaced and eventually be distributed randomly throughout the tumor.

## Summary

Ovarian tumors are induced in *Drosophila melanogaster* by ionizing radiation. A dose of 4,000r of $Co^{60}$ gamma rays produces an incidence of tumors 26 times the control rate. Whenever two or more tumors occur in the same ovariole they are adjacent to one another. This finding argues for a clonal origin of the tumors. A mechanism of tumor formation is postulated. It assumes that in a specific cytoplasmic zone of the germarium there is localized a chemical which interacts with a portion of the genome of the cells entering the region. The modified genetic material has a precise limitation placed upon its ability to permit further cytokinesis. Radiation occasionally inactivates that portion of the genome of an oogonium responsible for this interaction with the result that a limitation is not placed upon cytokinesis. This mutation is passed to the progeny of the oogonium, and tumors develop as a consequence.

## Acknowledgments

The author is grateful for the conscientious assistance performed by Mr. Jack Samuels. Thanks are also due to Dr. Malcolm Dole for providing the irradiation facilities.

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# THE INEFFECTIVENESS OF MEPROBAMATE ON GROWTH AND FEED ECONOMY OF WHITE LEGHORN COCKERELS

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(Received May 20, 1957)

It seems reasonable to expect that tranquilizing drugs might influence domestic animals in the same manner as reported for the human. It might logically follow that quieter, tranquil animals would expend less energy in movement, and nervous tension; react more favorably to social stress (e.g. peck order) etc., and consequently make more rapid and economical gains. This paper presents the results of a preliminary investigation designed to test the above hypothesis.

## PROCEDURE

Single Comb White Leghorn cockerels were selected as the experimental animal because they are inexpensive, grow rapidly, and are highly excitable. A common tranquilizing drug was observed to be meprobamate (2 methyl-2n-propyl-1, 3-propenediol dicarbamate). The meprobamate used in this study was obtained in the form of "Miltown", a proprietary compound, through a commercial source.

Meprobamate is commonly administered to humans at the level of 10 mg per pound of body weight daily. In this study an attempt was made to administer four levels: 0, 5, 10 and 15 mg per pound of body weight daily. Morrison (1956) provides an estimate of the age at various body weights and required feed for White Leghorn males. The average daily feed consumption and body weight for four age periods were estimated from Morrison's data and the amount of meprobamate per pound of feed necessary for each desired level calculated. The amount of meprobamate added per pound of feed for each level and period is shown in Table 1.

A commercial chick starter was fed the first 21 days and a commercial broiler ration the remainder of the experiment. All feed was in the form of a "meal" or "mash".

TABLE 1
MG Meprobamate Per Pound of Feed for Each of Four Periods

| Period: | Estimated mg Meprobamate per Pound of Body Weight per Day | | | |
|---|---|---|---|---|
| | 0 | 5 | 10 | 15 |
| 3–21 days | 0 | 29.76 | 59.52 | 89.29 |
| 22–35 days | 0 | 40.32 | 80.65 | 120.97 |
| 36–47 days | 0 | 67.21 | 134.41 | 201.62 |
| 48–54 days | 0 | 51.47 | 102.94 | 154.40 |

One hundred and four Single Comb White Leghorn cockerels were obtained at three days of age and randomly assigned to eight groups of thirteen chicks each. Two lots were randomly assigned each level. This represents a hierarchal or nested classification. This experimental design is amenable to analysis of variance which provides an estimate of variance components associated with both treatment and cage effects (Snedecor, 1956).

One bird was discarded prior to the start of the experiment and three birds were subsequently lost from another group as a result of leucosis. All other birds completed the experiment.

The birds were individually identified by wing bands and maintained in small electric brooders through twenty-one days of age. Thereafter, they were maintained in a standard eight cage broiler battery. The data include individual weights at the end of each period and feed consumption for each group for each period.

## Results and Discussion

The mean weights and feed economy by treatment and period are presented in Table 2. The data indicate the actual meprobamate consumption was very similar to the calculated dosage levels. The

TABLE 2
Mean Growth Rate and Feed Economy of Cockerels Receiving Various Levels of Meprobamate

| Age in days | Meprobamate, estimated mg/pound body weight, daily | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | 0 | | 5 | | 10 | | 15 | |
| | Wt. gm. | Gm. feed/ Gm. gain | Wt. gm. | Gm. feed/ Gm. gain | Wt. gm. | Gm. feed/ Gm. gain | Wt. gm. | Gm. feed/ Gm. gain |
| 21 | 178.1 | 2.79 | 176.6 | 2.82 | 184.0 | 2.80 | 184.1 | 2.80 |
| 35 | 409.6 | 2.56 | 423.1 | 2.41 | 420.0 | 2.61 | 413.0 | 2.92 |
| 47 | 646.8 | 2.65 | 655.7 | 2.70 | 653.9 | 2.79 | 660.3 | 2.87 |
| 54 | 792.7 | 3.58 | 806.8 | 3.46 | 807.9 | 3.53 | 808.0 | 4.01 |

analysis indicated a total absence of either treatment or cage effects. The feed economy data represent group means only and are not amenable to statistical tests of significance. It appears unlikely, however, that differences in feed economy exist.

Babcock and Taylor (1957) fed meprobamate to White Leghorn cockerels at the rate of 0.2, 0.4, 0.6, 0.8, 1.0, 1.4, 1.8, and 2.2 per cent of the diet.

They found no effect when the diet contained 0.4 per cent or less meprobamate but an inhibition of growth at higher levels. The highest level of meprobamate used in this experiment was slightly lower than 0.2 per cent of the diet. The results of this study support the findings and conclusions of Babcock and Taylor.

On the basis of these data and under the conditions of this experiment it seems reasonable to conclude that meprobamate has no influence on growth or feed economy of Single Comb White Leghorn cockerels when fed at the rate of 15 mg or less per pound of body weight daily. No attempt was made to induce stress during the experimental period. It is possible that the application of stress may affect birds treated with meprobamate differently than untreated controls.

## Summary

Meprobamate, a tranquilizing drug, was fed to White Leghorn cockerels at levels of 0, 5, 10 and 15 mg/lb. body weights. At these levels, this drug had no observable effect on the growth or well being of the experimental birds. These results confirm the findings of Babcock and Taylor.

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# THE PROPORTION OF LEUCINE AND LYSINE TO TRYPTOPHANE IN GROWING TISSUES[1]

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(Received August 13, 1957)

## INTRODUCTION

Implicit in the investigations of Osborne and Mendel was the conclusion, that not only was the presence of certain amino acids in the diet necessary, but also that the ratio between them was important. The results of their study (11) on the growth and maintenance of young rats, using diets containing gliadin or gliadin supplemented with lysine, indicated that the ratio of lysine to tryptophane required for growth is higher than that for maintenance. More recent results of other investigators have stressed the importance of dietary amino acid balance. Representative data of certain of these investigators, concerning amino acid requirements for maintenance and growth, are shown in Table 1. Their findings have been discussed in more detail in a previous publication (20).

In a preliminary study in our laboratory (19), the quantitative re-

TABLE 1
Dietary Requirement of Amino Acids

| | Tryptophane | Lysine | Leucine |
|---|---|---|---|
| | Maintenance Ratio | | |
| Adult rats[1] | 1.0 | 2.0 | 3.7 |
| Human adults[2] | 1.0 | 3.2 | 4.4 |
| | Growth Ratio | | |
| Repleting rats[1] | 1.0 | 5.4 | 6.1 |
| Human infants[3] | 1.0 | 5.6 | 14.0 |
| Human infants[4] | 1.0 | 3.0 | — |

[1] Steffee *et al.* (22).
[2] Rose (12).
[3] Albanese (1).
[4] Holt and Snyderman (8).

[1] This work was supported (in part) by research grant C-1370(C5) from the National Cancer Institute, Public Health Service. A preliminary report has appeared (18).

lationship of the amino acids tryptophane, lysine, and leucine, appeared to be significantly different in neoplastic and embryonic tissue from that in resting and regenerating liver. The present research has confirmed these findings and, further, has shown that the ratio of lysine to tryptophane, in particular, has a characteristic value range for "autonomous" tissues as contrasted to "controlled" tissues.

## Experimental

The following tissues were analyzed: normal, regenerating, tumor-bearing host, and maternal livers, diaphragm, intestinal mucosa, the sarcoma 180 (Crocker) from two sources, and the lymphosarcoma 6C3HED (Gardner). The normal tissues were from the Swiss mouse, the sarcomas were grown in the Swiss mouse, and the lymphosarcoma was grown in the C3H mouse. Table 2 summarizes the general information concerning each of these tissues. The tissues were analyzed individually or pooled into approximately equal groups.

After removal from the animals, the tissues were prepared according to the procedure of Schneider (15), and the washed residues brought to constant weight *in vacuo*. Portions of this material were taken for determination of the dry weight at 105° and of leucine, lysine, and tryptophane. Leucine and lysine were estimated microbiologically (7, 23), and tryptophane chemically (21). Microbiological assay of pure proteins gave reproducible values, quite comparable to those reported by other investigators, and there was no "drift" in the tissue values obtained at different assay levels. Chemical estimation of tryptophane in the presence of tissue (prepared as described above), with ratios of bound to free tryptophane of 3.7, 1.1, and 0.75, showed that the average recovery of tryptophane was 99.1%. All results are expressed on the basis of the material dried at 105°, on the assumption that it is pure protein.

## Results and Discussion

In Table 3 are given the analytical values for the various tissues which were studied. It may be seen that the tumors show a generally lower tryptophane content than do the normal tissues. This finding was not unexpected. Preliminary work in our laboratory (19), and the data of several other investigators (5, 9, 13, 14, also see 24), had indicated this result. The agreement with our own earlier findings

TABLE 2
General Information Relating to the Experiments

| Tissue | Age of Animals wks | Average Body Wt g | Organ Wt (Fresh) g | Sex | No. of Animals | No. of Groups | Remarks |
|---|---|---|---|---|---|---|---|
| Normal liver | 11-12 | 27.3 ± 1.6* | 0.99 ± .16 | F | 6 | 6 | Left lateral and median lobes of normal liver. |
| Regenerating liver | 11-12 | 25.6 ± 1.9 | 1.06 ± .11 | F | 5 | 5 | Liver residue, 96 hr after removal of left lateral and median lobes of liver. |
| Tumor-bearing host liver | 13-14 | 29.1 ± 2.3 | 1.68 ± .16 | F | 3 | 3 | Livers of animals sacrificed 10 days after grafting of sarcoma 180. |
| Maternal liver | 11-14 | 46.3 ± 3.3 | 2.61 ± .47 | F | 6 | 6 | Livers from pregnant animals. |
| Diaphragm | 6-7 | 23.9 ± 1.5 | —— | M | 42 | 4 | ——. |
| Intestinal mucosa | 6-7 | —— | —— | M | 46 | 5 | Consists of mucosa expressed after saline flushing of intestinal contents. |
| Sarcoma 180, Crocker (Clarke) | 7-8 | 20.4 ± 2.2 | 1.28 ± .40 | M | 4 | 2 | The tumors were excised 10 days after injection of the implant as a homogenate. |
| Sarcoma 180, Crocker (Bar Harbor) | 13-14 | 29.3 ± 2.0 | 0.33 ± .08 | F | 4 | 2 | The tumors were excised 10 days after implantation by trocar. |
| Lymphosarcoma—6C3HED, Gardner (Sugiura) | —— | —— | 1.88 ± .26 | M | 2 | 2 | The tumors were excised 10 days after implantation by trocar. |
| Embryos, mouse** | 8-22 mm (12-20 days) | 0.12 — 1.6 | —— | — | 52 | 10 | Whole embryos. |
| Embryos, chick** | 7-18 days | 0.76 — 22.9 | —— | — | 57 | 11 | Whole embryos. |

* Means and average deviation.

** Detailed information and analytical data on the chick and mouse embryos have been published previously (20), and are included herein in a composite form for comparison.

is particularly important, since in the former investigations a microbiological method was used for the determination of tryptophane, while in the present investigations, a chemical method was employed. Our data, in general, agree well with those of Mickelson and Barvick (10). However, in previous (unpublished) work in our laboratory, we have found no effect of a tumor on the tryptophane content of the host's liver at 5, 10, and 15 days after sarcoma 180 implantation. In the present work there may be evidence of a slight effect, but certainly no such decrease as the data of Mickelson and Barvick indicate, wherein the tumor-host's liver and tumor have the same content. Rosenbohm's data show that a lower range of tryptophane values is not an exclusive characteristic of tumors, being found also in certain (non-hepatic) normal tissues. It may be noted that the tryptophane values for the mouse embryo, as shown in our experiments, are similar to those found in the tumors.

Our data reveal that the lysine content varies little from one (mouse) tissue to another, in agreement with the findings of other workers (4, 14, 17). Mickelson and Barvick also find a constancy in the lysine content of the experimental livers and tumors but, in their study, normal liver has a lower value than any of the aforementioned tissues.

The leucine values are generally lower in the mouse embryo and in the tumors than in the various livers, diaphragm, and intestinal mucosa. Mickelson and Barvick also report a lower content of leucine in the tumors than in the tumor-host livers, but their value for normal liver is about the same as that in the tumors.

In the case of the chick embryo, each of the three amino acids has a higher value than that found in the mouse embryo.

As to the relationship between the three amino acids which we have determined (Table 3), the tissues fall into two groups. The "controlled" tissues exhibit a certain tryptophane : lysine : leucine ratio, which may be averaged to 1.0 : 3.9 : 5.6. The "autonomous" tissues evidence another pattern, which may be averaged to 1.0 : 6.0 : 6.8. The group to which a tissue belongs is more obvious when only the lysine to tryptophane ratio is considered. These values may be compared with the proportions found by the workers in the field of nutrition (Table 1). The patterns of the "controlled" and "autonomous" tissues compare well with those observed for "maintenance" and for

"growth". It should be noted however, that certain active tissues, which might have been expected to show a pattern similar to the "growth" pattern of the nutritionists, show instead a "maintenance" pattern. Isotope tracer experiments, for example those of Bloch (2) and Friedberg (6) have shown that intestinal mucosa is a very active tissue. The relative inactivity of diaphragm, and muscle in general, has been shown by Schoenheimer *et al.* (16) and Borsook *et al.* (3), among others. The analytically-determined ratios, however, permit of no distinction between intestinal mucosa and diaphragm on the basis of activity. Again, regenerating liver is very active, considered from the viewpoint of protein synthesis. This tissue also fails to exhibit any distinguishing pattern.

TABLE 3
Results of the Analysis of Various Tissues

| Tissue | Percent of Protein | | | Ratio | | |
|---|---|---|---|---|---|---|
| | Tryptophane | Lysine | Leucine | (Try | Lys | Leu) |
| "Controlled" | | | | | | |
| Normal liver | 1.38 ± .02* | 5.25 ± .1 | 7.0 ± .1 | 1.0 | 3.8 | 5.1 |
| Regenerating liver | 1.32 ± .01 | 5.3 ± .1 | 6.95 ± .1 | 1.0 | 4.0 | 5.3 |
| Tumor-bearing host liver | 1.29 ± .04 | 5.4 ± .1 | 7.1 ± .1 | 1.0 | 4.2 | 5.5 |
| Maternal liver | 1.31 ± .02 | 5.6 ± .1 | 6.95 ± .05 | 1.0 | 4.3 | 5.3 |
| Diaphragm | 1.19 ± .03 | 4.0 ± .3 | 7.5 ± .1 | 1.0 | 3.4 | 6.3 |
| Intestinal mucosa | 1.31 ± .06 | 5.2 ± .7 | 7.8 ± .1 | 1.0 | 3.9 | 6.0 |
| "Autonomous" | | | | | | |
| Sarcoma 180 (Clarke) | 1.05 ± .02 | 5.3 ± .2 | 6.65 ± .05 | 1.0 | 5.1 | 6.3 |
| Sarcoma 180 (Bar Harbor) | 0.84 ± .07 | 4.7 ± .1 | 5.6 ± .4 | 1.0 | 5.6 | 6.7 |
| Lymphosarcoma 6C3HED | 0.82 ± .04 | 5.6 ± .2 | 6.1 ± .0 | 1.0 | 6.8 | 7.4 |
| Mouse embryo | 0.82 ± .02 | 5.1 ± .1 | 5.9 ± .1 | 1.0 | 6.2 | 7.2 |
| Chick embryo | 1.24 ± .05 | 7.5 ± .2 | 7.7 ± .2 | 1.0 | 6.1 | 6.2 |

* Means and standard error.

## SUMMARY

The protein-bound tryptophane, lysine, and leucine have been determined for certain "controlled" [mouse] tissues, *viz.* normal, regenerating, tumor-bearing host, and maternal livers, intestinal mucosa, and diaphragm, and for certain "autonomous" tissues, *viz.* the sarcoma 180 from two different sources, and the lymphosarcoma 6C3HED. Previously published data on the mouse and chick embryos are included, because these embryos also represent "autonomous" growth.

When the tryptophane value is equated to unity, the "controlled" tissues show a characteristic pattern for the proportion, tryptophane : lysine : leucine, which is different from the characteristic pattern for the "autonomous" tissues. This distinction rests, mainly, on the lysine to tryptophane ratio.

## Acknowledgments

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# QUANTITATIVE GROWTH STUDIES WITH CHICK MYOBLASTS IN GLASS SUBSTRATE CULTURES[1]

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## INTRODUCTION

The use of established cell strains (e.g. HeLa, Strain L) has come to be regarded in recent years as a standard procedure for studies in tissue culture. Established strains do have obvious advantages in ease of cultivation and stability of growth patterns. Many have been cultured for years *in vitro* and will grow readily in simplified or semi-synthetic nutrients (Evans, Bryant, McQuilkin, Fioramonti, Sanford, Westfall, and Earle, 1956; Scherer, Syverton, and Gey, 1953; Chang, 1954; Eagle, 1955a). These characteristics have permitted the definition of amino acid, vitamin, and salt requirements for certain cell strains (Eagle 1955b) and the indefinite cultivation of at least one type (Strain L mouse fibroblast) in protein-free media (Evans et al., 1956).

On the other hand, it is increasingly clear that while reproducible information can be obtained on the properties of established strains, the significance of these facts for the growth of cells generally must be evaluated carefully. This seems particularly important if comparison or extrapolation is made to the corresponding cells *in vivo*. It should be emphasized that nearly all the established strains have originated by an alteration in type from the cells characteristic of the original population. The resulting stable cell lines may show metabolic as well as morphologic differences from the tissue of origin (Evans, Earle, Wilson, Waltz, and Mackey, 1952; Perske, Parks, and Walker, 1957). Although decisive experiments have not yet been reported, it appears that the ability of certain strains of cells

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to grow in protein-free media may be the result of mutation and selection. Cells freshly isolated from the intact organism do not grow progressively in the absence of protein. Finally, it is significant that all of the established strains studied so far have been found markedly abnormal with regard to chromosome numbers (Hsu and Moorhead, 1957). In the examination of four neoplastic and four normal strains, all proved to be heteroploid, often hypotetraploid, with very few or no diploid or tetraploid cells. It thus appears that variation is inherent in the properties of cell populations long isolated from the intact organism, and may indeed be a strategic step in the establishment of a stable pattern of growth, perhaps through the evolution of a heteroploid state or other cellular changes which may confer a selective advantage in culture.

These facts reveal the extent to which established strains may diverge from patterns characteristic of cells in the intact body, and emphasize the desirability of growth studies with freshly isolated cells as well as with established cell lines. Such studies have been carried out with fresh explants cultivated in plasma ever since the early days of tissue culture (Parker, 1950). This technique is not suited to quantitative procedures, however, owing to the difficulty in separating cells and medium. The more useful monolayer cultures on a glass substrate, first introduced by Earle and associates (Evans and Earle, 1947; Evans, Earle, Sanford, Shannon, and Waltz, 1951) have been employed extensively for growth studies, but for the most part with established strains (Sanford, Waltz, Shannon, Earle, and Evans, 1952; Sanford, Westfall, Fioramonti, McQuilkin, Bryant, Peppers, Evans, and Earle, 1955; Parker, Healy, and Fisher, 1954). Recently, however, modifications of the monolayer technique have come into wide use for the propagation of virtues (Dulbecco, 1955) and the results obtained indicate abundantly the stability of primary monolayers under appropriate conditions.

The present experiments were designed to explore the use of myoblasts recently isolated from chick skeletal muscle as a standardized system for quantitative growth studies in monolayer cultures. Chick cells are especially useful for such studies in view of the ready availability of materials, including homologous media if needed. An extensive literature is also at hand on the growth and nutrition of chick cells as studied by older methods (see Murray and Kopech, 1953). Emphasis in the present experiments has been placed on the use of

growth rates as a means for characterizing and comparing cell populations under study. Logarithmic growth rates (Monod, 1949) provide a more intrinsic measure of the growing system than do maximal cell counts or arbitrary points along a growth cycle, as commonly employed by other investigators.

## Materials and Methods

Cell populations for experiment were obtained by standard procedures to be outlined in detail in the experimental section. In brief, this involves a preliminary cultivation of fresh explants in plasma to obtain a large population of dispersed cells, which are then released by trypsin from the plasma clot and inoculated as a monolayer in stock cultures. The resulting populations are again harvested with trypsin and used for specific assays or experimental studies.

Both plasma cultures and monolayers were maintained in media made up primarily from serum and embryo extract. Plasma was drawn as needed by heart puncture from young cockerels. Sera were obtained commercially (Difco and Cappel Laboratories). Significant differences were found in individual lots of serum, as detailed in a later section. Embryo extract was prepared by homogenizing 10-day chick embryos in an equal volume of Earle's solution as described in a preceding paper (Harris, 1952). Earle's solution was employed for the saline fraction of the culture media. In this fraction were incorporated routinely 0.002% phenol red, 100 units/ml sodium penicillin G (Merck) and 50 units/ml dihydrostreptomycin (Pfizer). Trypsin solutions for subculturing or cell counts were prepared by dissolving trypsin 1-300 (Nutritional Biochemicals Corp.) in Puck's solution (Puck, 1956). This solution (designated Saline A by Puck) has the following composition:

| | |
|---|---|
| NaCl | 8.00 gm |
| KCl | 0.40 gm |
| $NaHCO_3$ | 0.35 gm |
| Glucose | 1.00 gm |
| Phenol red | 0.02 gm |
| Glass distilled water | 1000 ml |

For cultivation of glass substrate cultures the T flasks described by Earle and Highhouse (1954) were found most useful. Plasma cultures were maintained in Carrel D-3.5 flasks. All cultures were incubated at 38°C.

## EXPERIMENTAL

### *Culture techniques and growth measurements*

*Preparation of standard cultures*—Strains of chick myoblasts were routinely isolated from 15 day embryos by the following procedure. Explants (ca. 2 mm diameter) were cut from freshly excised leg muscle in Earle's solution and inoculated singly into Carrel flasks. The medium consisted of clotted chicken plasma with a supernatant containing 10 EE 20 HS[3]. Further details on the preparation of plasma cultures may be found in an earlier paper (Harris, 1952). After incubation at 38°C for 10 days the outgrowths were removed with a platinum spatula into Petri dishes with Earle's solution. The central fragments and peripheral plasma were trimmed away with Bard-Parker scalpels, No. 10, and the residual cell sheets transferred to a centrifuge tube. Warm trypsin (0.1% in Puck's solution) was added using 0.5 ml per outgrowth and the stoppered tube incubated at 38°C. After 45 minutes with intermittent agitation by tapping a turbid suspension was obtained, although shreds of the original outgrowths remained intact. After centrifuging (1000 RPM for 5 minutes in an International Centrifuge, type SB) the supernatant was discarded and nutrient medium added without washing the cells. Small amounts of trypsin remaining were inactivated by the medium and produced no detectable effects. The cells were suspended and the remaining shreds broken up by further pipetting. Clumps and particulate material were removed by passage through a platinum filter. This unit included two sieves of 80 and 150 mesh respectively according to the original design of Evans et al. (1951), but was modified by converting the upper reservoir into a short open funnel which was covered with a Petri dish. The stopcock beneath the sieves was removed. The final suspension consisted of single cells and was counted by the standard procedure described in the next section. Approximately 1.0-2.0 x $10^6$ cells were obtained for each original outgrowth in a Carrel flask. Typically the cells were spherical and intact, with no evidence of damage from the trypsin treatment.

From this initial suspension, stock cultures were prepared by

[3] For simplicity in designating the composition of media used in the present investigation, the percentages of serum and embryo extract are shown with abbreviations HS = horse serum, CS = chicken serum, and EE = embryo extract (prepared by the usual 1:1 dilution with saline). The remainder of the medium, not indicated in the formula, is in all instances Earle's solution.

inoculating 3.0 x $10^6$ cells into 8.0 ml nutrient medium of desired type, usually 10 EE 20 HS, in a T-60 flask. The cultures were gassed with 5% $CO_2$ in air and incubated for three days with a 50% fluid change each day. The cells adhered to the floor of the flask and formed a continuous sheet by the third day, although not yet at maximal numbers.

Stock cultures incubated for three to four days were used for all experiments. In harvesting the cells, medium was removed and four ml warm 0.1% trypsin added. The flasks were incubated 10 minutes, after which gentle agitation was sufficient to loosen portions of the cell sheet still remaining on the glass surface. After transferring the suspension to a centrifuge tube, the cells were sedimented at 1000 RPM for 5 minutes. The supernatant was discarded and the pellet resuspended in nutrient medium appropriate to the experiment. The suspension was finally clarified by passage through a platinum filter, counted, and the concentration adjusted on this basis with blank medium to give 1.0 x $10^6$ cells/ml.

Experimental T-15 flasks were inoculated with 0.2 ml of the counted suspension, using 1.5 ml graduated pipettes. The stirring apparatus designed by Earle and associates for L cells (Evans et al., 1951) did not give satisfactory results with freshly isolated chick cells, apparently damaging these more delicate materials by mechanical agitation. As the data in subsequent sections will indicate, however, replicate cultures can also be obtained by the simpler pipetting procedure specified above. Each T-15 flask was filled with 1.8 nutrient medium before inoculation with cells, giving a final volume of 2.0 ml. The cultures were gassed with 5% $CO_2$ in air and incubated at 38°C. Preliminary studies indicated that for these preparations of chick myoblasts, a daily fluid change of 50% was optimal for maintaining stability of cells and fluid phase. This procedure was adopted uniformly in all experiments to be described.

*Procedure for cell counts*—Cell numbers were determined at intervals during the growth cycles by direct counts on trypsinized suspensions from experimental flasks, which were sacrificed for this purpose. Ordinarily three flasks were pooled for a single determination. The medium was removed and the flasks drained briefly on absorbent tissue, after which an appropriate volume of freshly prepared trypsin solution was added to each flask with a chemical pipette (1.0-4.0 ml, according to the expected cell number). The concentration of trypsin

was 0.25% for cultures incubated from one to four days, increasing to 1.0% for older cultures where separations were more difficult. The T-15 flasks with trypsin were incubated for 10 minutes, agitated gently, and examined microscopically for removal of cells and residual clumping. The suspension in each flask was pipetted repeatedly with a 1.5 ml pipette, washing all surfaces and dispersing aggregates, until microscopic observation showed essentially a single cell suspension. Continued careful pipetting was essential to break up the tough cell sheets from older cultures in serum-embryo extract. The final suspensions from the three flasks were pooled and separate pipette samples transferred rapidly by hand to a total of four chambers in two standard hemocytometers (bright-line, double improved Neubauer ruling). The unstained, intact cells were counted with a 10x objective and 15x wide field oculars. Individual cells were discrete and easily recognized for counting without the necessity of releasing nuclei or even staining. Ordinarily a total of 1000 or more cells were counted in three or four hemocytometer chambers, choosing an appropriate number of large (1 mm) squares arranged symmetrically within individual chambers. The average count per square was determined and used to calculate the total number of cells per flask. For convenience and accuracy in counting, the optimal cell concentration appeared to be 75-125 cells per 1 mm square in the counting chamber. Errors in counting increase with larger cell numbers, while with more dilute cell suspensions it is necessary to count more than four chambers to complete a standard sample of 1000 cells.

The reliability of this procedure was examined statistically for a series of cell counts taken at random from laboratory records. Table 1 gives a summary of this data. For each cell count a coefficient of variation was computed from the formula $Cv = s/m$, where $s =$ standard deviation and $m =$ average count per hemocytometer square. The percentage error was calculated from the procedure followed by Sanford, Earle, Evans, Waltz, Shannon (1951)

$$\text{Percentage error} = \frac{k\ Cv}{\sqrt{n}}$$

where $k =$ a constant defining the percentage of times on the average a specified error or less will be obtained ($k = 1.96$ at the 95% level), and $n =$ number of hemocytometer squares counted.

TABLE 1
Variation in hemocytometer counts on trypsinized suspensions of chick cells cultivated in horse serum-chick embryo extract

| Count | Total cells counted | Hemocytometer squares counted | Average count per square | Observed variance | Coefficient of variation | Percent error (95% level) |
|---|---|---|---|---|---|---|
| 1 | 722 | 36 | 20.1 | 24.2 | 0.244 | 8.0 |
| 2 | 2042 | 24 | 85.1 | 139.0 | 0.139 | 5.6 |
| 3 | 1800 | 24 | 75.0 | 75.0 | 0.116 | 4.6 |
| 4 | 648 | 24 | 27.0 | 56.8 | 0.278 | 11.1 |
| 5 | 2025 | 24 | 84.4 | 185.0 | 0.161 | 6.4 |
| 6 | 1213 | 36 | 33.7 | 54.2 | 0.217 | 7.1 |
| 7 | 776 | 36 | 21.6 | 38.5 | 0.287 | 9.4 |
| 8 | 1147 | 12 | 95.6 | 181.0 | 0.141 | 7.9 |
| 9 | 641 | 36 | 17.8 | 21.1 | 0.258 | 8.4 |
| 10 | 1076 | 24 | 44.8 | 48.7 | 0.154 | 6.2 |
| 11 | 2064 | 24 | 86.0 | 55.5 | 0.086 | 3.4 |
| 12 | 1662 | 18 | 92.3 | 179.0 | 0.145 | 6.8 |
| 13 | 1926 | 12 | 161.0 | 234.0 | 0.095 | 5.3 |
| 14 | 1583 | 24 | 66.0 | 81.2 | 0.136 | 5.5 |

As shown in Table 1, the error in counting trypsinized cell suspensions was well below 10%, 95% of the time, for samples of 1000 cells or more. Slightly greater fluctuation occurred in samples of 500-1000 cells. According to previous studies, the number of cells falling within an area of designated size in a hemocytometer follows the Poisson distribution, for which the variance is typically equivalent to the mean (Snedecor, 1956). The observed variances in the present experiments were higher than the theoretical values, averaging 1.5 times the mean for the observations listed in Table 1. This may reflect slight residual clumping in the final cell suspensions. Both variance and percentage error in the present experiments, however, were remarkably similar to the values reported by Sanford et al. (1951) for population estimates of L cells derived from nuclear counts. It thus appears that counts based on trypsinized suspensions afford a reproducible estimate of the cell populations under study.

*Cell counts vs DNA analysis*—Although the foregoing data indicate that direct counts in trypsin give stable estimates of cell number, the question arises whether substantial numbers of cells from culture flasks are destroyed during the trypsin treatment, thus introducing a significant error. An independent population estimate is available through determination of DNA, following the original observation of Boivin, Vendrely and Vendrely (1948), since extended by many other

investigators (see Alfert and Swift, 1953) that the DNA content is essentially constant for the cells of a given species. Accordingly, DNA determinations were made on a number of myoblast cultures in T-15 flasks, and correlated with cell counts made in parallel on similar flasks of the same series. Analysis for DNA followed the method of Ceriotti (1952) with modifications based on the procedure of Bonting and Jones (1957). After removal of the medium the culture flasks were rinsed gently with saline and drained on absorbent tissue. To each flask 0.6 ml 1 N-NaOH was added, the flasks stoppered and incubated overnight at 38°C. Each DNA determination was based on the pooled digests from four culture flasks, giving duplicate samples of 1.0 ml. The indole reaction and chloroform extraction were carried out in 12 ml centrifuge tubes, and the final color read at 490 mμ using a Beckman model DU spectrophotometer, with cuvettes (Pyrocell Mfg. Co., New York, N.Y.) modified for a sample volume of 0.8 ml.

Table 2 gives a comparison of cell counts and DNA values in two different experiments, using standardized preparations of chick myoblasts as outlined in preceding sections. While the DNA/cell ratios show some scatter, the data between the two experiments are in good agreement. The average value of $3 \times 10^6$ μgms DNA/cell is substantially similar to the values previously reported for chick cells in terms of DNA phosphorus (Davidson, Leslie, Smellie, and Thomson, 1950; Healy, Fisher, and Parker, 1954). The consistency of these results indicates that direct counts in trypsin afford a valid estimate of chick cell populations under the conditions noted.

TABLE 2

DNA content of chick myoblasts in glass substrate cultures

| Cell strain | Incubation, days | Cell count per flask x $10^6$ | DNA per flask, μgm. | DNA/cell, μgm. x $10^{-6}$ |
|---|---|---|---|---|
| | 1 | 0.18 | 0.69 | 3.8 |
| | 2 | 0.39 | 0.97 | 2.5 |
| 377 | 3 | 0.54 | 1.71 | 3.2 |
| | 4 | 0.72 | 3.33 | 4.6 |
| | 5 | 1.14 | 3.92 | 3.4 |
| | | | Average | 3.5 |
| | 3 | 1.78 | 4.99 | 2.8 |
| 738 | 4 | 2.06 | 7.29 | 3.5 |
| | 5 | 2.70 | 8.79 | 3.3 |
| | | | Average | 3.2 |

*Determination of growth rates*—The principal data to be presented are based on measurements obtained from cultures in logarithmic growth phase. In practice cell counts made at intervals were plotted against hours of incubation on a semilogarithmic grid and the period of linear increase identified (see next section). The position of the growth line for this data can be established graphically, or by a short statistical procedure devised by Kermack and Haldane (1950), and simplified by Imbrie (1956). This procedure gives slope and intercept for the growth line in log phase.

From the established growth line the logarithmic growth rate can be calculated from the relation

$$k = \frac{\log_2 X_2 - \log_2 X_1}{T_2 - T_1}$$

where $X_1$ and $X_2$ are any two points on the growth line at times $T_1$ and $T_2$ respectively. This constant represents the average number of generations per cell per unit time (Monod, 1949). In practice it is convenient to use common logarithms to base 2. This is accomplished by the incorporation of a conversion factor into the expression of growth rate

$$k = 3.32\ \frac{(\log X_2 - \log X_1)}{T_2 - T_1}$$

If the growth line used is obtained by the statistical method outlined by Imbrie (1956), the logarithmic growth rate is $k = 3.32\ a$, where $a$ = the calculated slope of the growth line (reduced major axis) expressed in common logarithms of cell count/unit time.

Alternatively, increase in cell number can be expressed in terms of mean generation time (MGT). This represents the interval required for doubling the population and is the reciprocal of the logarithmic growth rate

$$MGT = \frac{1}{k}$$

The mean generation time can also be obtained by graphical methods from the growth line for log phase. This involves determining the time interval between any two points on the line which are separated by a factor of two.

Although the principal focus of interest in the present experiments

lies in logarithmic growth rates, some estimates were also made of growth rates during the phase of declining growth, and of maximal cell populations for individual experiments. The procedures employed by Brody (1945) for increase in body weight during the phase of negative growth acceleration gave a good fit to certain data which will be described in the following section. The appropriate rate factor (—K) should not be confused with the logarithmic growth rate, and here connotes a constant logarithmic decline in growth rate until a limiting population (A) is reached. Graphical methods for the estimation of —K and A are given in the publication of Brody cited above.

## Kinetic Studies with Chick Myoblasts

*Typical growth curves*—A number of growth cycles were followed for different strains of chick myoblasts growing in 10 EE 20 HS. Two representative curves of those obtained are shown in Fig. 1. The more characteristic result is illustrated by Strain No. 471, which follows the classic pattern of initial lag period, well-defined logarithmic growth phase, and final approach to a limiting population. The mean generation time of approximately 27 hours is a typical figure for myoblasts in this medium. During the phase of declining growth, the rate of population increase declined logarithmically as expressed by the rate factor $-K = 0.012$. This means that the difference in cell count at any time from the final limiting population was reduced at a constant rate of approximately 1% per hour.

Variation in growth patterns of individual strains of myoblasts was observed, as Parker (1932, 1933a, 1933b) showed earlier for chick fibroblasts growing in plasma cultures. The mean generation time during log phase varied from 21 to 36 hours for specific strains, although reproducible in separate experiments done simultaneously with the same strain. In some instances (e.g.—strain No. 745, Fig. 1) a well-defined log phase was not observed, with a logarithmic decline in growth rate from the outset.

At the microscopic level, chick myoblast cultures developed as a uniform network of cells with the usual morphology of fibroblasts. When the cell sheets became continuous, however, the differentiation of muscle fibers in small numbers could be observed grossly. Uniform population growth was obtained only with glassware scrupulously cleaned, and with nontoxic sera. A number of batches of horse serum

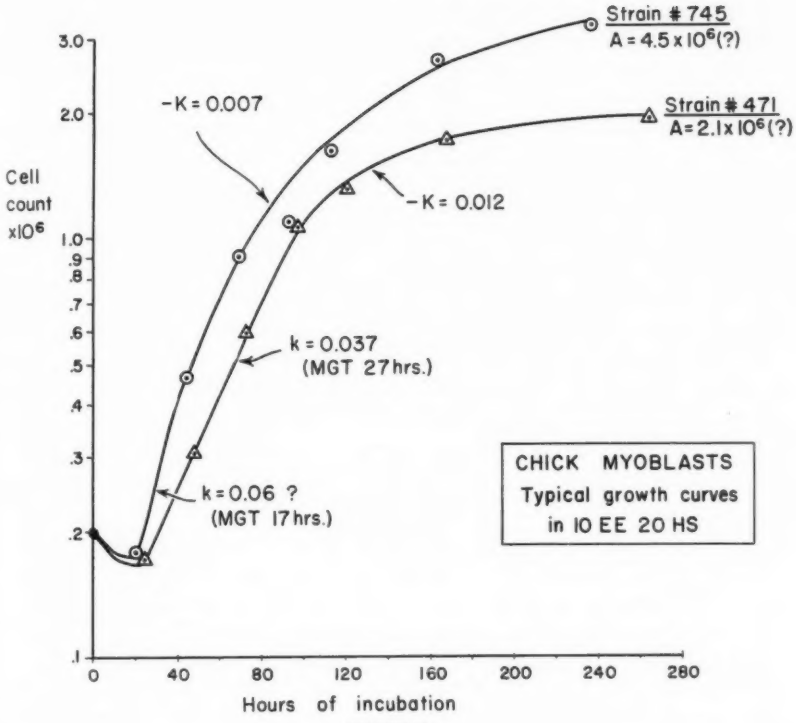


FIGURE 1

obtained commercially were unsuitable for the cultivation of chick cells, causing excessive vacuolation and clumping or balling of cells, or even the collapse of cell sheets after a few days of cultivation.

*Effects of embryo extract and serum*—The use of logarithmic growth rates to characterize the growth of populations can be further illustrated by experiments designed to compare the relative effect of embryo extract and serum in the stock medium described above.

In an initial series, cultures were established in 5% and 15% embryo extract ($EE_{50}$) respectively, holding the concentration of horse serum constant at 20% as before. Cultures in 15 EE grew much more rapidly, with cells smaller and more uniform than in populations maintained with 5 EE. The difference in growth rates is

shown in Fig. 2. It is apparent that the logarithmic growth rate of chick myoblasts is proportional to the concentration of EE. Similar results were obtained with other strains of myoblasts, although the absolute level of growth varied with the individual strain.

In a second series of experiments the concentration of EE was held at 10% and the level of HS in the medium varied. Below 10 HS stable sheets of cells did not develop. Populations in 10 EE 10 HS were similar in appearance and grew at the same rate as cultures in 10 EE 20 HS. In 10 EE 40 HS growth was inhibited somewhat with cell morphology variable and abnormal. These results are documented by the growth curves shown in Fig. 3. It appears that the logarithmic growth rate of chick myoblasts is not influenced by the concentration of horse serum, except for inhibition at higher levels.

Since this result might be due to the use of heterologous rather than homologous serum, further experiments were performed with

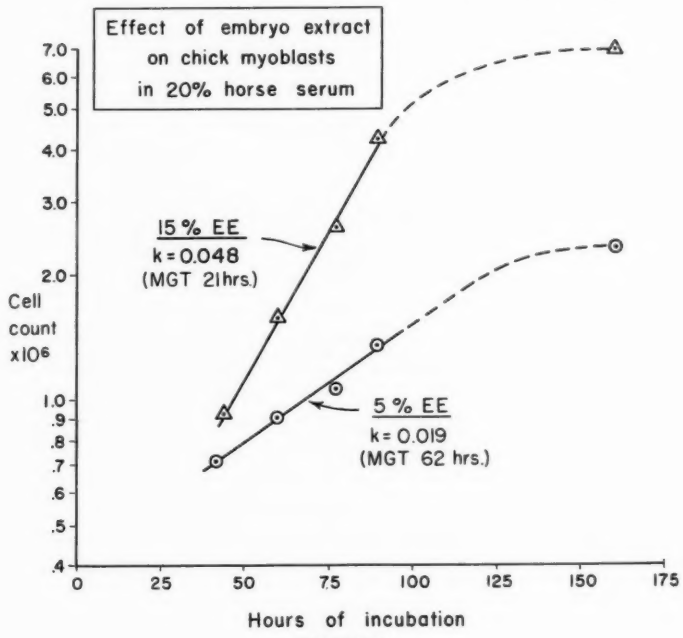


FIGURE 2

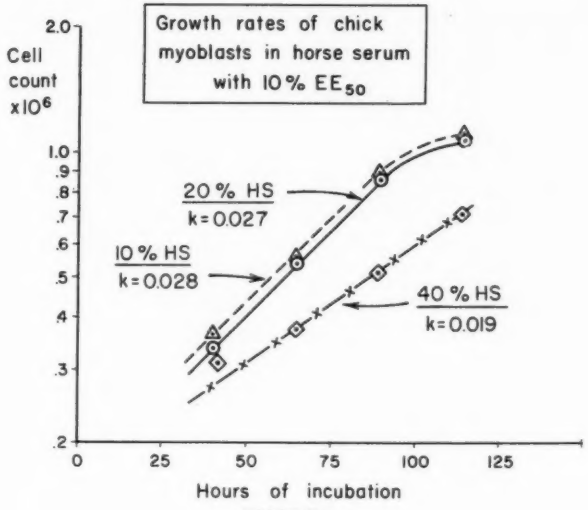


FIGURE 3

chicken serum in place of horse serum in the culture medium. Culture of myoblasts in EECS were at first difficult to maintain. The initial cell suspensions adhered normally to the culture flasks and proliferated uniformly for two or three days, after which there was a netlike aggregation of cells which soon sloughed off the glass. This phenomenon appeared to reflect increased tryptic activity in the cell sheets in EECS and could be prevented completely by the addition of crystalline soybean trypsin inhibitor (Worthington) to the culture medium at 0.1 mg/ml. All further experiments in EECS included trypsin inhibitor.

Figure 4 gives the results of an experiment in which the concentration of CS was varied between 10% and 40%. Although this cell strain did not show clear-cut logarithmic growth, it is apparent that with chicken serum as with horse serum, the concentration of serum used does not determine the growth rate of myoblasts except for inhibition at higher levels.

The question arises whether inhibition with higher levels of either homologous or heterologous serum is caused by serum proteins or by smaller inhibitors. In this connection aliquots of chicken serum were

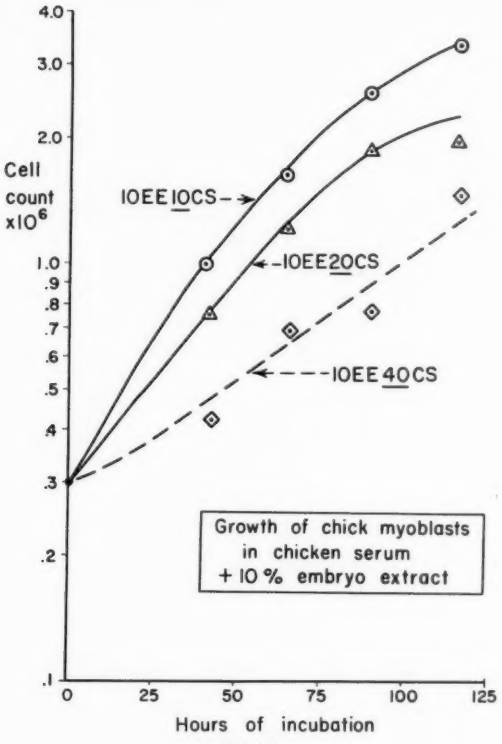


FIGURE 4

dialyzed for three days with constant agitation against several changes of buffered saline. The resulting dialyzed serum was used to establish cultures in 10 EE 10 CS and 10 EE 40 CS respectively. As with the undialyzed sera, the series at the higher serum level showed a lowered growth rate and microscopic signs of inhibition. These results suggest that the inhibitors in question are nondialyzable, or are closely associated with nondialyzable components.

*Long-term cultivation of myoblasts*—It should be emphasized that the various strains of myoblasts employed for kinetic studies were all obtained by the standard procedure outlined in a preceding section and were used at the same time interval after isolation from the

embryo. The further evolution of cell strains was followed by serial subculture of the primary cultures on glass. At the end of each growth cycle the cells were harvested with trypsin, resuspended in 10 EE 20 HS, and inoculated into fresh T60 flasks at 1.0 x $10^6$ cells per flask. The cultures declined progressively in growth rate and total yield, and became static after about 8-10 passages so that the strain could no longer be maintained. During this period the cells gradually lost the uniform, clearcut appearance of primary cultures, becoming ragged and heterogeneous, with large amounts of intercellular debri. Stained preparations showed great variation in the appearance of individual cells, with minute forms as well as increasing numbers of cells with very large, irregular nuclei. Preliminary DNA analysis suggested that the average value per cell in old cultures was 3-4 times the amount found in freshly isolated strains (see p. 156).

It seemed possible that the decline of cell strains in subculture might result from progressive damage by the trypsin used to harvest the cells at the end of each growth cycle. To test this possibility, myoblasts were subcultured serially without trypsin, by vigorous shaking to dislodge portions of the cell sheets, which could then be used to start cultures of the next passage. Although this procedure was successful, the same progressive changes in cells were noted, and the cell strain eventually lost. These results indicate that damage to cells by trypsin was not a limiting factor in the declining vigor of older cultures.

Other cultures were carried serially in EECS rather than EEHS. In the presence of homologous serum deterioration in serial subculture was less marked but the cell populations eventually became heterotypic and static as described above. It appears, therefore, that myoblasts freshly isolated from chick embryos will not proliferate indefinitely as monolayers in serum-embryo extract media. The mechanism of decline is not immediately apparent but the increasing amount of extracellular material, particularly in the immediate vicinity of cells in older cultures, suggests progressive loss or extrusion of essential cell components (e.g.—cytoplasmic proteins). Conceivably this process might be linked to an increasing frequency of endomitosis (Stillwell, 1952; Hsu and Moorhead, 1956) and other mitotic aberrations, leading ultimately to a heterotypic cell population and cessation of proliferation. Doubtless altered strains of chick myoblasts could be found which would grow indefinitely in the media used here, but none was observed in the present experiments.

## Summary

1. Experimental procedures are described for the routine isolation of myoblasts from chick embryos, as a standardized system for growth studies in monolayer cultures. The use of freshly isolated cells is emphasized as a means of minimizing changes by mutation and adaptation, which appear to be characteristic of established cell strains maintained for long periods in tissue culture.

2. Appropriate methods are outlined for kinetic studies based on growth rates rather than the conventional comparison of maximal population levels. Logarithmic growth rates, or mean generation times during log phase, appear to represent the most intrinsic parameters of growth for cell populations.

3. The logarithmic growth rate of chick myoblasts in a serum-embryo extract medium is proportional to the concentration of embryo extract. Homologous or heterologous serum is required for stable growth but has no direct influence on growth rate except for inhibition at higher concentrations.

4. Myoblasts isolated directly from chick embryos do not show stable growth indefinitely, when maintained as monolayers in serum-embryo extract. Over 8-10 serial passages the growth rate diminishes, the cell populations become heterotypic, and the cell strains are eventually lost. An explanation is suggested based on the progressive loss of essential cell components and increasing incidence of mitotic aberrations.


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# GROWTH OF THE MARSUPIAL *TRICHOSURUS VULPECULA* AND A COMPARISON WITH SOME HIGHER MAMMALS

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## INTRODUCTION

The brush-tailed possum (*Trichosurus vulpecula* Kerr) is a typical marsupial in that the young are reared within a pouch, after being born at a stage of development which in many aspects would be considered pre-natal for higher mammals. For studies on growth, hair follicles, etc. this is a most desirable feature, as development in a pouch, unlike development *in utero*, lends itself both to observation and experiment. For such purposes a breeding colony of *T. vulpecula* was established in this laboratory. Records were kept of the body weights and of certain linear body dimensions of the young, born or reared in the colony.

The purpose of the present paper is to analyse these data, together with a set of measurements on Tasmanian pouch-young. This has been done in the light of current concepts on growth as outlined in a symposium under the leadership of Zuckerman (1950). Comparisons have been made with higher (eutherian) mammals using data published or made available to us. In addition, criteria for age determination and information on breeding seasons, ultimate sizes, etc. are extracted from the data.

## MATERIAL AND METHODS

The basis of this study consists of observations on 110 preserved Tasmanian pouch-young (see Appendix) and periodic measurements on 72 living specimens from near Sydney, New South Wales (see Map, Fig. 5), of which 16 were born in captivity and 13 were pouch-young when collected (Appendix, Table I). The remainder (24 fe-

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FIGURE 1

*Trichosurus vulpecula*. Pouch-young (T32, ♀) at 5 ± 4 days after birth. Mouth of mother's pouch opened for photographic purposes.

FIGURE 2

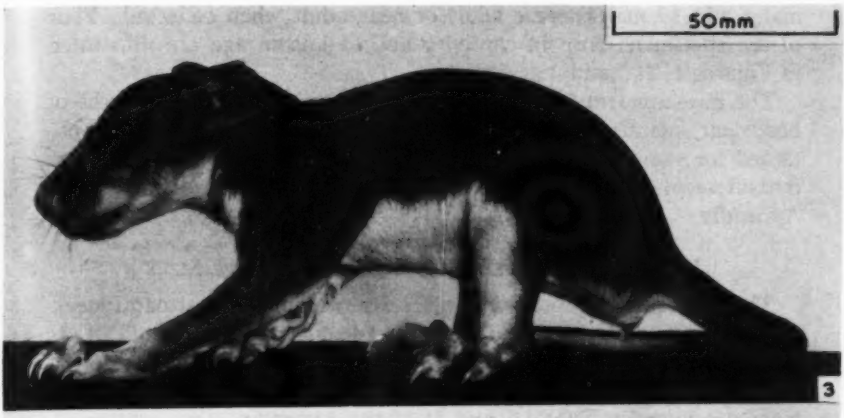
*Trichosurus vulpecula*. Pouch-young (T27, ♂) at 36.5 ± 3.5 days after birth. The young, which is still attached to the teat, has been taken out of the pouch for photographic purposes.

20 mm
1

20 mm
2

FIGURE 3
*Trichosurus vulpecula*. Advanced pouch-young (T20, ♀) when first seen out of the pouch at 115.5 ± 3.5 days after birth.

FIGURE 4
*Trichosurus vulpecula*. Adult (T26, ♂) at 413.5 ± 3.5 days.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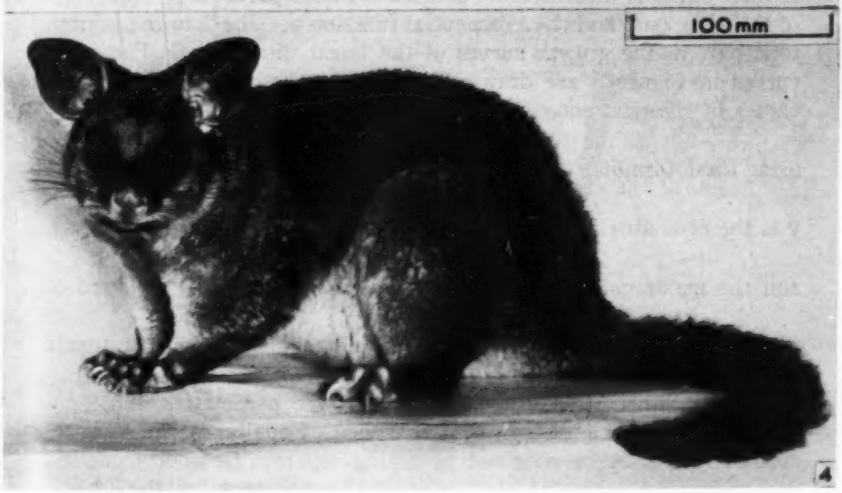
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males and 19 males) were adult or near adult when collected. Four of the specimens born in captivity and of known age are illustrated in Figures 1, 2, 3 and 4.

The measurements made were the body weight, and the lengths of head, ear, manus, pes and tail. Crown-rump lengths were also obtained for some small pouch-young. The manner in which the linear measurements were taken is illustrated in Figure 5, and defined in the Appendix.

## Individual Growth Curves and Ultimate Sizes

The observations plotted in Figure 5 represent the post-natal measurements on two typical individuals (T4 ♂, unknown age; T18 ♀, known age) representing an autumn birth in the wild and a spring birth in captivity, respectively. The data for these and other specimens are given in Tables I and II of the Appendix.

The sigmoid pattern of the plotted observations (Fig. 5) is evident but their relatively small scatter generally showed up conspicuous deviations from attempted fittings of either logistic or Gompertz curves to these data. It was therefore decided to fit the parabola $p + qt + rt^2$ to the early part and the exponential function $a + be^{-kt}$ to the asymptotic part of the growth curves of the linear dimensions. The fitted curves in Figure 5 are drawn according to these formulae and the values of the parameters are given in the Appendix (Table II). By using these formulae the specific growth rate (i.e. $\frac{1}{y}\frac{dy}{dt}$ or $\frac{dlny}{dt}$ where y is the ordinate) is found to be decreasing over the range examined, and the maximum for the absolute growth rate (i.e $\frac{dy}{dt}$) is found to occur close to where the parts concerned reach half their ultimate sizes.

The functions fitted, each containing three parameters, must not be given too much biological significance. Equally satisfactory results may usually be obtained by fitting separate Gompertz, logistic or exponential curves to the lower and upper parts of the growth curves. These curves are discussed by Medawar (1945) and further comments and details of fitting will be given in a subsequent paper

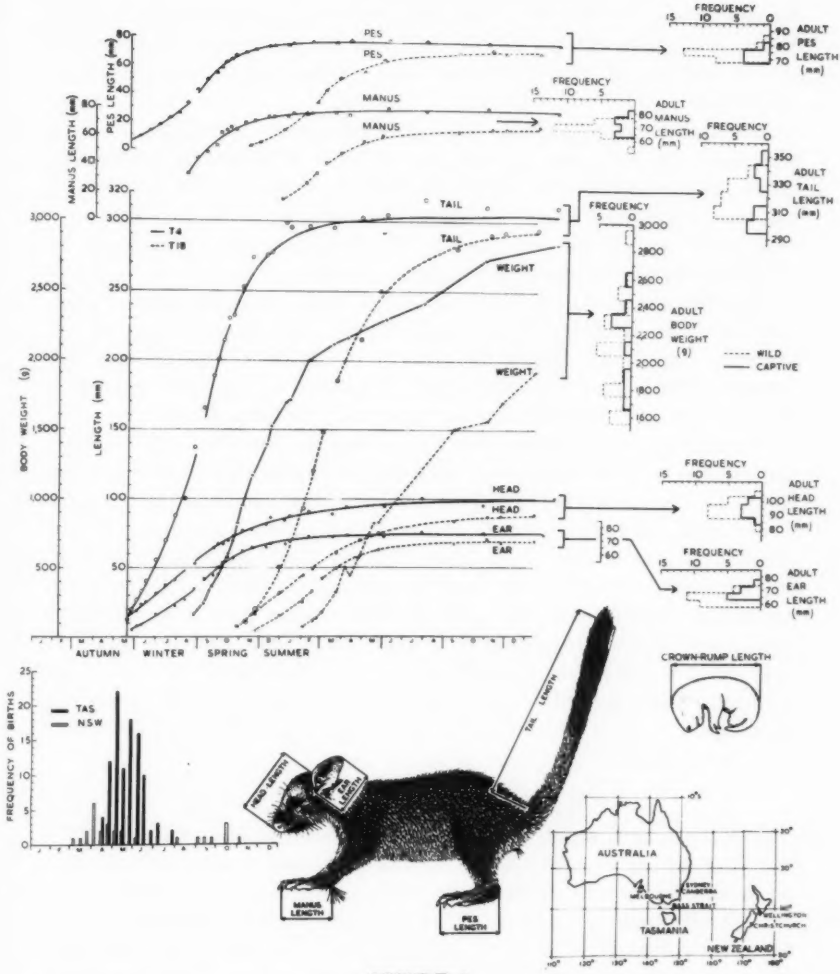



FIGURE 5

*Trichosurus vulpecula* showing: methods of taking measurements, individual growth curves for two individuals, histograms on ultimate sizes for wild and captive specimens, histogram on seasonal distribution of births in Tasmania and New South Wales, and map showing localities mentioned in the text.

by one of us (A.M.W.V.). An alternative is to fit growth curves by polynomials (Bunak, 1946) but this leads to unrealistic deformations in the calculated growth curves and to laborious computing procedures.

The body weight, unlike skeletal dimensions, is subject to little observational error and can actually decrease as is sometimes observed when the pouch-young is separated from its mother. This is probably one of the reasons why the simple functions fitted to the linear observations would not adequately describe the trend displayed by the body weight observations shown in Figure 5.

The histograms on the right of Figure 5 are ultimate sizes of parts measured, as obtained from adult N.S.W. specimens (nine of the animals (Appendix, Table I) reared in captivity and 24 captured animals, adult when first observed). There is no apparent difference between the histograms for these wild and captive animals.

No periodic measurements of any marsupial from birth to maturity are available in the literature. The few available short-term observations of Tyndale-Biscoe (1955) on the growth in length of the head and crown-rump of four captured and recaptured pouch-young of *T. vulpecula* in New Zealand (see Map, Fig. 5) show no differences from the present observations on captive animals. Dunnet's (1956) linear measurements of three non-captive pouch-young from near Canberra (see Map, Fig. 5) are made in a different manner and are therefore not strictly comparable with ours or Tyndale-Biscoe's. However, Dunnet's body weight measurements of nine juveniles, at estimated ages from 77 to 177 days after birth, are generally consistent with our observations on captive animals.

## Age Determination

For concurrent studies on hair development by one of us (A.G.L.) the need arose for methods of age determination. For this purpose all available measurements from birth onwards of 12 animals born in captivity and of known age (± 3.5 days or less, Appendix, Table I), are plotted in Figure 6. As separation of these animals from their mothers may affect growth to some extent, any subsequent measurements were disregarded for purposes of age determination. Measurements in 10 or 20 day intervals were averaged both for time and dimension and the results were plotted as open circles in Figure 6. Linear interpolation between these points was then used to construct

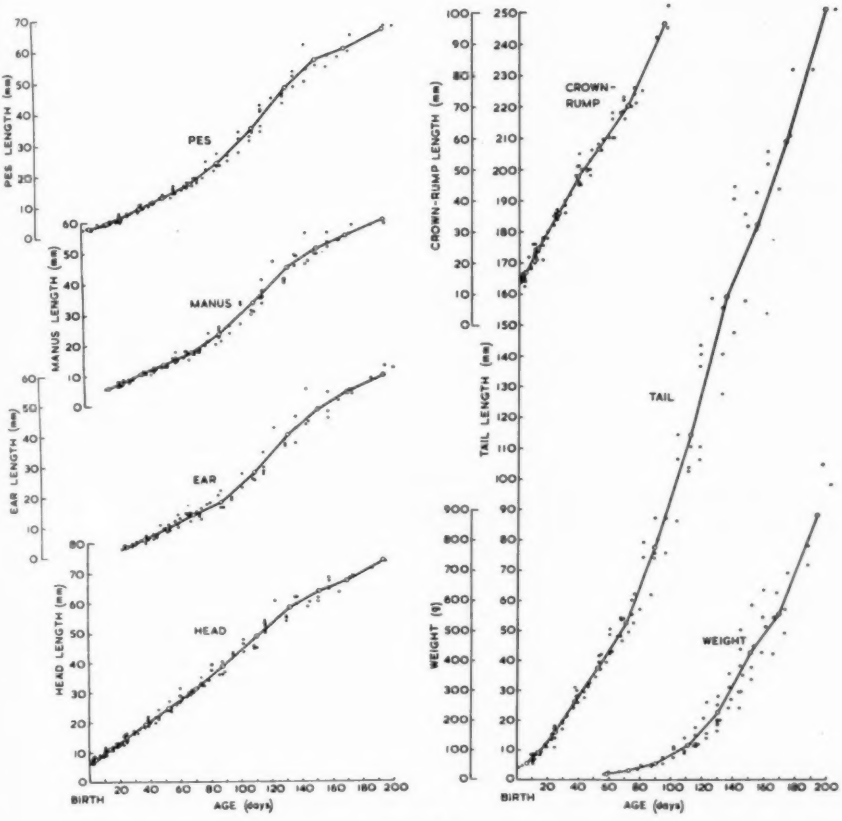
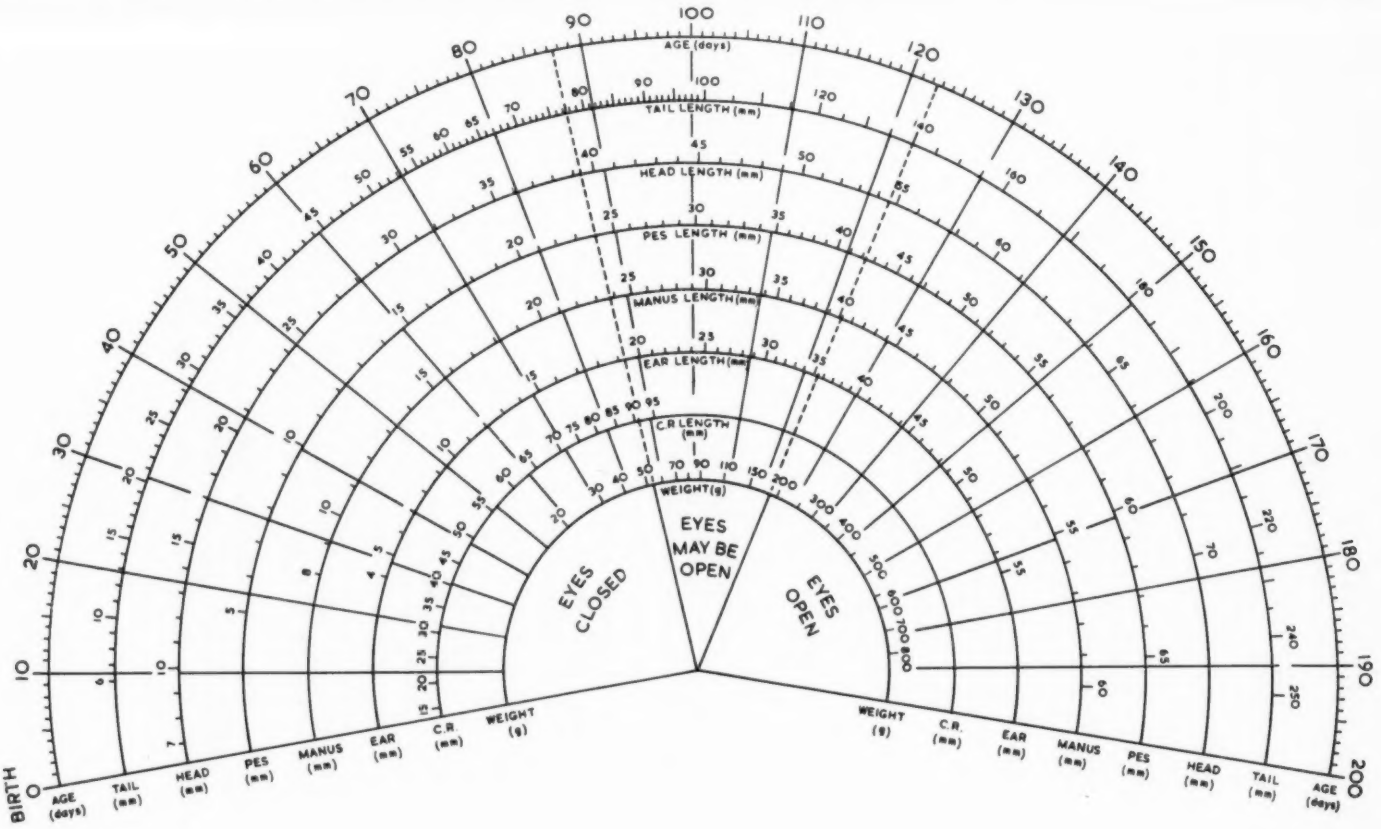



FIGURE 6

*Trichosurus vulpecula*. Periodic measurements on 12 specimens of known age (± 3.5 days). For average points (o————o) see text.

FIGURE 7

*Trichosurus vulpecula*. Nomogram for determining age and missing dimensions from given ones, constructed from the data of Figure 6.


AGE (days)
TAIL LENGTH (mm)
HEAD LENGTH (mm)
PES LENGTH (mm)
MANUS LENGTH (mm)
EAR LENGTH (mm)
C.R LENGTH (mm)
WEIGHT (g)
EYES CLOSED
EYES MAY BE OPEN
EYES OPEN
BIRTH
AGE (days)
TAIL (mm)
HEAD (mm)
PES (mm)
MANUS (mm)
EAR (mm)
C.R (mm)
WEIGHT (g)
WEIGHT (g)
C.R. (mm)
EAR (mm)
MANUS (mm)
PES (mm)
HEAD (mm)
TAIL (mm)
AGE (days)

a nomogram for age determination (Fig. 7). Each circular scale in this figure represents a dimension measured, and points on a common radius represent expectations of these measurements for any given age. By reading this nomogram along a radius each dimension will lead to an estimate of age. The scatter shown in Figure 6 indicates that the head length is the most reliable measurement; it has the advantage of being easily taken, of having the largest age range and the smallest variation.

## Seasons of Birth

The above technique of age determination was then applied to 100 of the juvenile Tasmanian specimens (the details of which are deposited in the McMaster Laboratory Library, C.S.I.R.O., Sydney), and 13 N.S.W. specimens of unknown age (Appendix, Table I). The calculated dates of birth, together with the dates of birth for 15 N.S.W. specimens of known age (Appendix, Table I), are presented in a histogram (Fig. 5).

On this basis it appears that the mode of the distribution of autumn births for the Sydney area is about one month earlier than that for Tasmania. No Tasmanian spring births were apparent, because nearly all Tasmanian specimens were collected from June to August (i.e. before Spring).

The present observations on the seasons of birth of the 29 N.S.W. specimens (Appendix, Table I) are similar to those of Bolliger (1940) who says that "Around Sydney the main breeding season is at the beginning of autumn (March-April) and the majority of fully grown females seem to get pregnant about this time. A minor breeding season takes place in the early spring (August-September), when probably much less than half of the females become pregnant. On rare occasions one also finds young animals which, according to their age, must have been conceived in between the two breeding seasons".

Dunnet (1956) records two main breeding seasons for the Canberra area but finds no intermediate breeding. Tyndale-Biscoe (1955), in New Zealand, found births in almost every month of the year but with a distinct peak in the autumn (April-May).

## Early Post-Natal Body Weight Related to Age

Periodic body weight measurements of living pouch-young cannot conveniently be carried out because they are attached to a teat.

However, one set of measurements (preserved body weight, and the lengths of head, ear, manus, pes and tail) was available for each of 62 of the Tasmanian pouch-young. By using the nomogram technique for determining the ages of these specimens from their linear dimensions, it was possible to plot their body weights as a function of age. These preserved body weights and estimated ages were then averaged over 10 day intervals and the results were plotted (Fig. 8). For comparison the early weights and averages of the N.S.W. specimens of known age, as obtained in Figure 6, were also plotted in Figure 8. There is no apparent difference between these two body weight-age relations.

## Relative Growth

The shape of a juvenile animal is not in general the same as that of an adult and it is this change of proportions of the various parts that forms the subject of "relative growth". The coherence of the growth of different parts of the same animal has given rise to much study and speculation regarding general laws presumed to relate the growth of the parts to each other and to the body as a whole.

In looking for likely formulae to relate two parts, x and y, one may think of a multiplicative growth process (e.g. yeast in which new material reproduces) and measure its activity by $\frac{1}{y}\frac{dy}{dt}$. If we now suppose that there is a constant ratio between the growth activities (specific growth rates) of two parts, x and y, we have $\frac{1}{y}\frac{dv}{dt} = k\frac{1}{x}\frac{dx}{dt}$ which is equivalent to the parobolic relation (1) $y = bx^k$, or to $\log y = \log b + k \log x$, a linear relation between log y and log x (Huxley, 1932). (k = ratio of the specific growth rates, i.e. the slope of the relative growth curve when plotted logarithmically.) If however we think of the growth process as being of a more additive type (bone, claws, hair, etc.) its activity is more appropriately assessed by $\frac{dy}{dt}$ and we could put forward the hypothesis that the growth rates of two

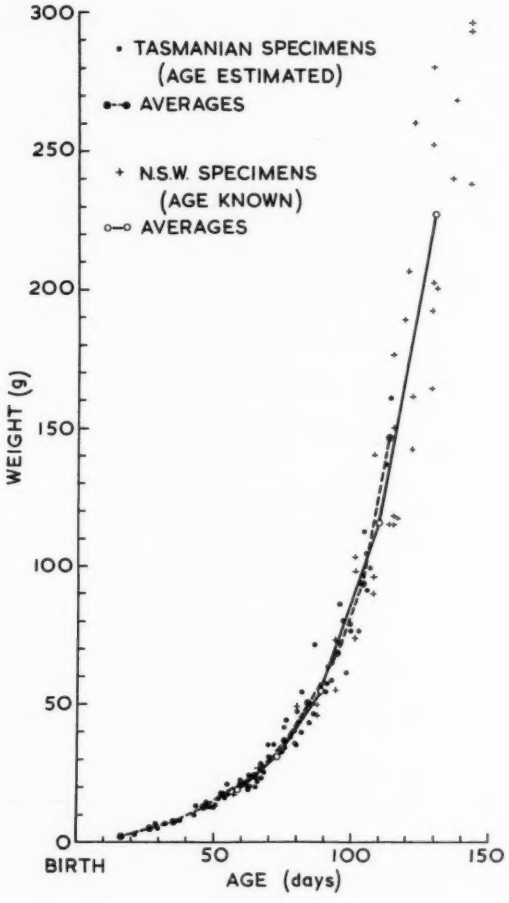



FIGURE 8

*Trichosurus vulpecula*. Preserved body weights of 62 Tasmanian pouch-young as a function of estimated age, and live weights of N.S.W. specimens as a function of known age. For further details see text.

parts are proportional, i.e. $\frac{dy}{dt} = m \frac{dx}{dt}$ which is equivalent to (2)

$y = m\,x + c$, a linear relation. ($m$ = ratio of the absolute growth rates, i.e. the slope of the relative growth curve when plotted arithmetically.)

Formulae (1) and (2) represent easily understood growth processes and can be plotted linearly. Deviations from linearity are conspicuous and these formulae can therefore conveniently be used as standards by which to study relative growth. If the parts of an animal were maintaining their proportions from a certain stage onwards, relative growth diagrams when extended backwards should form a straight line passing through the origin, a special case of both formulae (1) and (2) when $k = 1$ and $c = 0$. The mathematical implications of using formulae (1) and (2) will be discussed in a subsequent paper by one of us (A.M.W.V.).

The number of relative growth diagrams that can be constructed from two and five dimensions is one and 10 respectively and from N dimensions is given by the Newtonian coefficient $\binom{N}{2}$. We do not propose to explore all these possible cases. The diagrams in Figure 9 were prepared with a view of examining what kind of growth relations might actually exist between various parts of the body. It is interesting to note that at least some of the observed trends in these relative growth diagrams cannot be adequately represented by formula (1) as advocated by Huxley (1932, 1950) because it implies one-way curvature. Neither can some of these diagrams be represented by formula (2) because there is an obvious non-linear trend. This means that simple hypotheses, such as the ratio of the absolute growth rates or specific growth rates being constant, are not always tenable. Examples of the use of formula (1) are more plentiful than those for formula (2). However, D'Arcy Thompson (1952, p. 208) finds many cases in which a linear relation is more appropriate. Shepherd, Sholl and Vizoso (1949) find a linear relation between the length of leg, forearm, jaw and height for man. A further example of a straight line relation can be constructed from Cloete's (1939) data on prenatal sheep by plotting forelimb length against hindlimb length.

The relative growth diagrams (Fig. 9) were also used for comparing N.S.W. and Tasmanian specimens of *T. vulpecula*. On the whole there is no conspicuous difference between these geographically isolated populations (see Map, Fig. 5), although there is a tendency

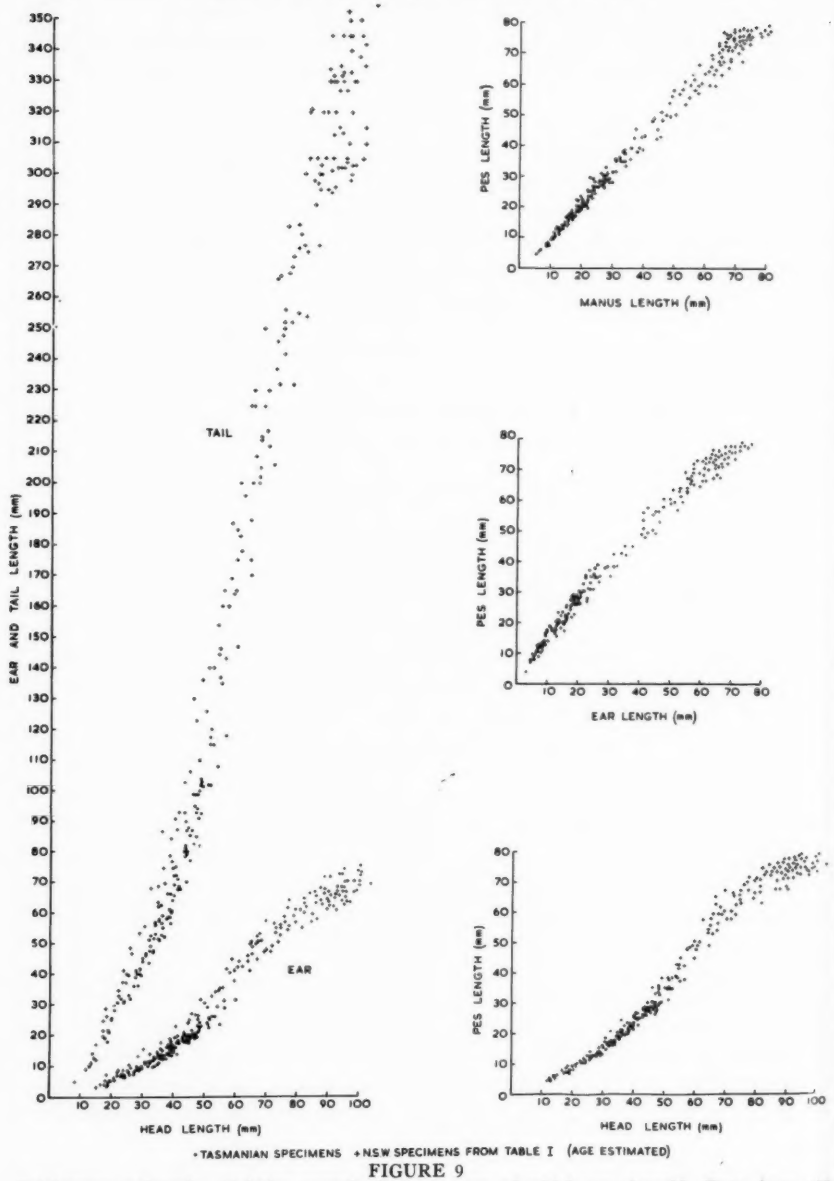



FIGURE 9

*Trichosurus vulpecula*. Relative growth diagrams, two of which are sigmoid. Data from 109 Tasmanian pouch-young and consecutive measurements on 13 N.S.W. specimens born in the wild (See Appendix, Table I).

for the points representing Tasmanian observations to be lower in plots involving head length as abscissa. This is most evident when ear length is plotted against head length. Unfortunately, it has not been possible to study this more closely because the observations on the Tasmanian specimens refer only to pouch-young and the largest recorded head length is approximately 60 mm. Le Souef (1916) noticed that some populations of *T. vulpecula* (e.g. from Deal and Flinders Island in Bass Strait (see Map, Fig. 5)) have slightly smaller ears in proportion to their size than those from the mainland, but are otherwise similar.

## Linear Equivalence

In addition to comparing various parts of the body amongst themselves we now propose to study their growth relative to some standard. Rather than give the status of a standard to a particular skeletal part (which possibly had different functional significance for different species) we shall compare various parts with the body as a whole. For this purpose we introduce the concept *"linear equivalence"* which is defined as *the length of the side of a cube whose volume is equal to that of the animal.*

In practice the volume for an animal is not readily obtained but in most cases it is adequate to work with the cube root of the body weight and assume that the specific weight of the animal is one. For example, if the body weights of two animals are 1000g and 1g then their linear equivalences are 10 cm and 1 cm respectively.

To study the relation between a part and the whole of the body the linear dimensions of *T. vulpecula* were plotted against the corresponding linear equivalences (Fig. 10). Where the slope $m$ (defined in formula (2)) is maximal the part is growing at a maximum rate relative to the body as a whole.

The slope of the straight line obtained by joining a point of the diagram (Fig. 10) with the origin equals the *relative magnitude* (i.e. $\frac{\text{linear dimension}}{\text{linear equivalence}}$) of the part concerned. By moving this point along the diagram we obtain a qualitative picture of how the relative magnitude changes. Although our data are concerned with post-natal growth only it is clear that during the period of foetal development the relative magnitude is zero before the part appears. Further, it

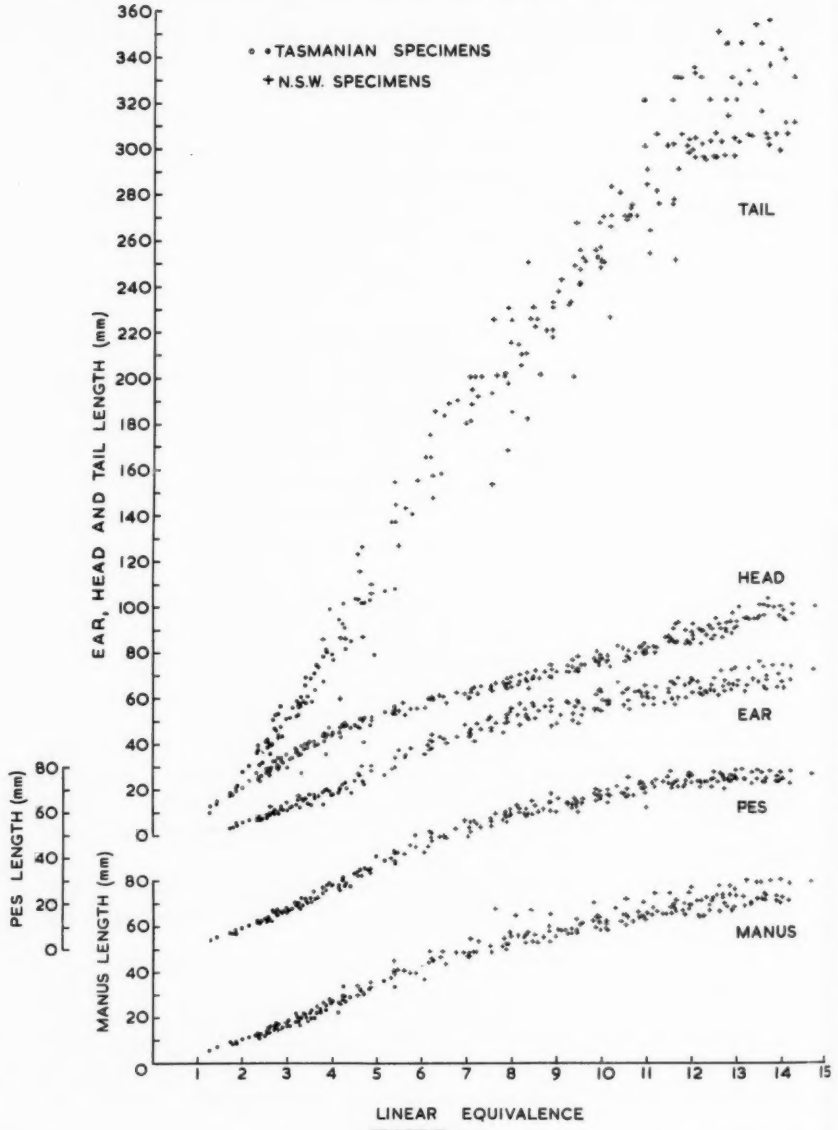



FIGURE 10

*Trichosurus vulpecula*. Linear dimensions plotted against linear equivalence ($\sqrt[3]{\text{body weight}}$). Data from 68 Tasmanian pouch-young and consecutive measurements on 13 N.S.W. specimens.

will be seen from Figure 10 that a maximum relative magnitude occurs in all the parts studied and that this appears to be reached earliest in the head.

Note that by using the cube root of the body weight we need not use log transformations for making weights and linear dimensions comparable, a procedure which was also used by Latimer (1932) in his study on the limbs of pre-natal cats. Pütter (1920, p. 325) referred to cube root of the weight as the "ideale Längeneinheit".

It is of some interest to study how linear equivalence compares to an over-all measure of size, such as crown-rump length. For this purpose Dick's (1956) data on forehead-rump length of foetal sheep were plotted against linear equivalence and this resulted in a straight line passing through the origin. Joubert's (1956) data on the crown-rump length of foetal sheep gave similar results.

Once a proportionality between a part of the body and linear equivalence is established this part may then be used to compare other parts and to study the body weight condition of the animal.

## Comparison with Other Marsupials

To our knowledge very few studies on linear growth and changes in body weight for marsupials are available in the literature. Hill and Hill (1955) present linear and weight observations for the native cat (*Dasyurus viverrinus*) for the period of pouch life. No periodic measurements were made by these authors, but as far as their data go they show the same general features as the present observations for *T. vulpecula*. In these studies of *T. vulpecula* no records were kept of the total length of this animal. However, Hartman (1928) made length and body weight measurements during the post-natal life of the American opossum (*Didelphis virginiana*). If the total length and corresponding weight is read from the smooth curve through his data (pp. 174-175) and the total length is plotted against the linear equivalence, a straight line relation is found, and one may expect such a relation to hold for *T. vulpecula*.

Reynolds (1952) measured only one character (snout-rump length) of *Didelphis* and only during the period of pouch life, but the data do show the curvature typical of the lower part of generally sigmoid growth curves.

Observations by Lyne (unpublished data) on relative growth in the bandicoot (*Perameles nasuta*) during the entire post-natal period show essentially the same qualitative picture as *T. vulpecula*.

## Comparison with Higher Mammals

As linear size differences are probably more readily appreciated than weight differences it was decided to compare various mammals (man, cow, sheep and mouse) with *T. vulpecula* in terms of their linear equivalence (i.e. a linear measure of overall size). Moreover, the cube root reduction of the body weight, like a log transformation, facilitates graphical representation and has the added advantage of remaining a readily understandable measure of size.

Figure 11 was constructed for the purpose of studying, on absolute scales, the early post-natal growth of *T. vulpecula* in relation to the embryonic and further development of some higher mammals. It must be noted that the specimens from which the pre- and post-natal curves are plotted may not be strictly comparable as they were taken from different strains or breeds within the species.

The gestation period of *T. vulpecula* was conveniently taken to be 20 days since, in the present study, one instance of a birth with a gestation period of not more than 21 days has been recorded. There is, however, some evidence that the period of uterine development is approximately 16 days (Bolliger and Carrodus, 1940).

It is perhaps rather unexpected that up to 40 days from conception, the mouse is heavier than the other species examined, and the sheep, during its pre-natal development, is larger than the cow (Fig. 11). It may also be noted that up to about 70 days, *T. vulpecula* is larger than man. There seems to be no systematic relation between the relative foetal and ultimate sizes.

With the aid of equivalent scales the growth curve for *T. vulpecula* is compared with those for man, cow, sheep and mouse (Fig. 12). The abscissa indicates percentage *"half-time"*, i.e. *the time taken to reach half the ultimate linear equivalence when measured from conception,* and the ordinate is in terms of the percentage ultimate linear equivalence. This makes all the curves go through the half-way point, i.e. the point with coordinates 100 and 50. It is now clear that the various growth curves do not have the same shape and that they are not symmetrical with respect to the half-way point. This, incidentally, shows that, in general, fitting of symmetrical growth curves, such as the logistic, is not appropriate. If the growth curves were symmetrical they would go through the point with co-ordinates 200 and 100.

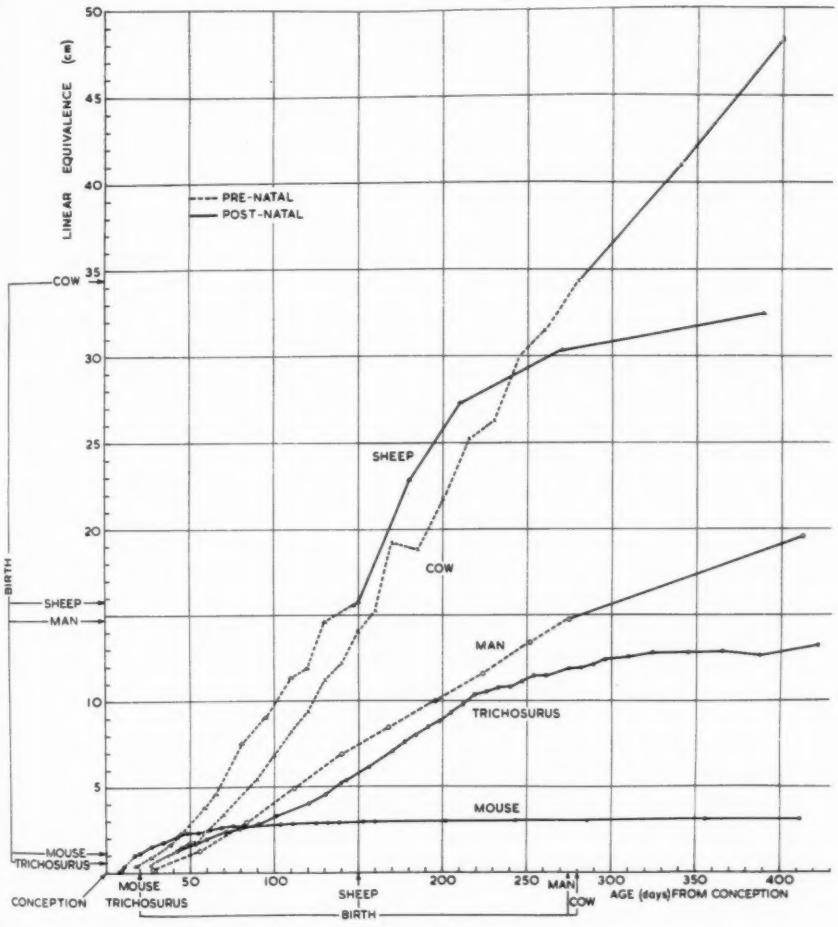



FIGURE 11

Comparison of the post-natal development of *T. vulpecula* with the embryonic and further development of man, cow, sheep, and mouse in terms of linear equivalence ($\sqrt[3]{}$body weight). Arrows indicate positions of births on both linear equivalence and time scales. At birth *T. vulpecula* and mouse are comparable in size (for further details see text). Sources of the data are given in the Appendix.

The generally sigmoid nature of the growth curves in Figure 12 is evident and, for *T. vulpecula,* this may even be observed in the post-natal period.

Other features to be noted in this comparison are that *T. vulpecula* shows the fastest maturation, and the growth curve for man differs conspicuously from those of the other mammals examined, both before and after the half-way point.

Another way of comparing the growth curves of different species is to consider conception and 98 per cent of the mature body weight as biologically equivalent points for different species (Brody, 1927, p. 7). However, for the purpose of qualitative comparisons the present method is preferred. Furthermore, due to the flatness of the growth curve in the asymptotic part, the 98 per cent point cannot be determined with great accuracy and the half-way point, which is in a steeper (if not the steepest) part of the growth curve, is more satisfactory in this respect.

In Figure 12 the points at which births occur are marked by arrows on both the horizontal and vertical scales and they show up clearly the relatively early birth of T. vulpecula. Note that the birth of this marsupial occurs at less than 10 per cent of its ultimate linear equivalence whereas in the higher mammals examined it occurs at between 35 and 45 per cent. The table in Figure 12 presents the half-times and ultimate linear equivalences of the mammals examined.

## Summary

A study has been made of the post-natal linear dimensions and body weights of the 182 specimens of *Trichosurus vulpecula*—110 from Tasmania and 72 from New South Wales, of which 16 were born in captivity and 13 were pouch-young when collected. Formulae are given for the individual growth curves of two specimens and a nomogram for age determination, based on 12 such individual records from birth to approximately 200 days, has been constructed and used for preparing a histogram on seasons of birth. Relative growth diagrams are constructed and they clearly show that the formulae derived for the simple additive and multiplicative growth processes may not give an adequate description of the data. These diagrams were also used to compare two isolated populations of *T. vulpecula.*

The concept *linear equivalence* (cube root of the body weight) is

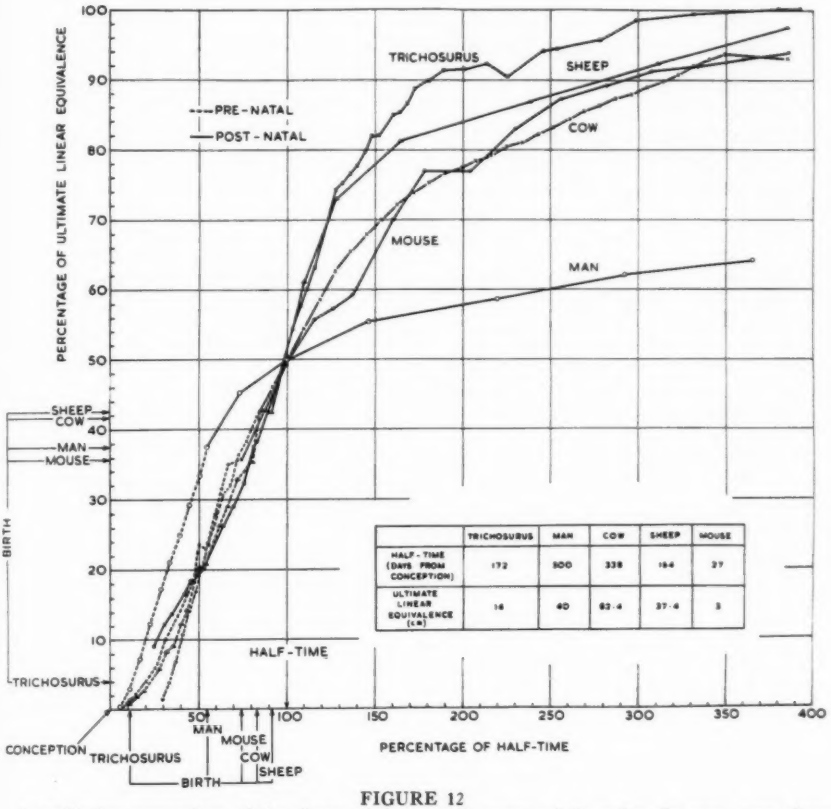



FIGURE 12

A qualitative comparison of the slopes of the growth curves of *T. vulpecula*, man, cow, sheep and mouse from conception onwards with the aid of equivalent scales (see text). Note that the estimated percentage ultimate linear equivalence for *T. vulpecula* at birth is 4 per cent, whereas, for the higher mammals examined it is over 35 per cent. Sources of the data are given in the Appendix.

introduced and used as an overall measure of size against which the parts of the body are compared and also as a standard for studying differences within and between species.

Based on linear equivalences a qualitative comparison of the growth curves (from conception onwards) of *T. vulpecula*, man, cow, sheep and mouse, has been made using both absolute and percentage scales. The relatively early birth of *T. vulpecula* is evidenced by the post-natal part of the growth curve being large compared with that for the higher mammals examined.

## Acknowledgments

The assistance of many people in the collection of material in Tasmania and New South Wales is gratefully acknowledged. The data on the Tasmanian material were obtained by one of us (A.G.L.) while in receipt of a Commonwealth Research Grant. We are indebted to Dr. J. Pearson (then Director) of the Tasmanian Museum, Hobart, for permission to examine material in his care, and to Mr. P. G. Schinckel, Sheep Biology Laboratory, C.S.I.R.O., Prospect, N.S.W., for permission to publish his observations on South Australian Merino sheep. Miss A. G. Culey, of the McMaster Laboratory, Professor K. W. Cleland and Dr. H. O. Lancaster, of the University of Sydney, and Mr. P. G. Schinckel kindly read the manuscript. Also, we are indebted to the following people for technical assistance: Miss A. M. Bartle, Miss A. J. Bathgate, Mr. T. Dagg, Miss M. J. Heideman, Mr. I. T. Roper and Mr. J. Stone. The assistance of Mr. J. C. Coles and Miss B. Miller in preparing the text-figures for publication is gratefully acknowledged.

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## APPENDIX

*Material and Methods*

The length measurements illustrated in Figure 5 are defined as follows:

*Head*—from the tip of the snout to the occiput, measured between two parallels; *ear*—from its most distal point to the base of the inferior notch; *manus*—from the proximal margin of the ulnar carpal vibrissa-protuberance to the tip of the longest digit, not including the claw; *pes*—from the point of the heel to the end of the longest digit, not including the claw; *tail*—from its tip (excluding hair) to its dorsal base; *crown-rump*—from the crown to the rump, measured between two parallels.

Unlike the linear measurements the live weights of the specimens shown in Table I were obtained at intervals only after the period of attachment to the teat. The remainder of the N.S.W. specimens were measured and weighed when collected and periodically in captivity.

Sixty-nine (32 females and 37 males) Tasmanian pouch-young collected by one of us (A.G.L.), were taken from freshly caught animals in various localities during 1949-50 and 1952-53. Nearly all of these specimens were fixed in Bouin's fluid in the field and stored in 70-80 per cent alcohol. The length measurements and weights of these specimens were recorded after fixation. The remainder of the Tasmanian pouch-young material (21 females and 20 males), preserved in alcohol or formalin and also collected in various parts of Tasmania, was measured at the Tasmanian Museum, Hobart, during 1952.

*The sources of data used in the comparison of the growth of T. vulpecula with some higher mammals*

| | | |
|---|---|---|
| *T. vulpecula.* | *Pre-natal.* | No material available. |
| | *Post-natal.* | From 17 to 114 days after birth (ages estimated).—Averages obtained from 62 Tasmanian pouch-young. From 122 days after birth to maturity (age estimated).—Specimen No. T4 (see Table II). |
| *Man.* | *Pre-natal.* | Scammon (1942, p. 30). |
| | *Post-natal.* | Scammon (1942, p. 32) and Brody (1927, p. 6, Fig. 1a). |
| *Cow.* | *Pre-natal.* | Winters, Green and Comstock (1942, pp. 36-37). Foetuses from grade beef heifers and grade cows of mixed breeding. |
| | *Post-natal.* | Eckles (1920, p. 11). Normal Holstein females, from birth to 30 months. Brody and Ragsdale (1923, p. 19, chart 12). Holstein females, from 34 to 82 months. |
| *Sheep.* | *Pre-natal.* | Cloete (1939, p. 557). South African Merinos. |
| | *Post-natal.* | South Australian female Merinos. (Unpublished data collected by Mr. P. G. Schinckel, Sheep Biology Laboratory, C.S.I.R.O., Prospect, N.S.W.). |
| *Mouse.* | *Pre-natal.* | MacDowell, Allen and MacDowell (1927, p. 62). |
| | *Post-natal.* | Robertson (1916, p. 369). Males. |

TABLE I

Details of All New South Wales Specimens of *Trichosurus Vulpecula* Born in Captivity or Pouch-Young When Collected

| Specimen No. and Sex | Date when first observed | Age (days) when first observed | | Age (days) when last observed | Age (days) when Eyes | | Age (days) when first seen out of pouch | Age (days) when separated from mother |
|---|---|---|---|---|---|---|---|---|
| | | Age known* | Age estimated† | | Last seen closed | First seen open | | |
| T3 ♂ | 6.v.54 | — | 31 | 59 | 59 | — | — | — |
| T4 ♂ | 20.v.54 | — | 24 | 1004 | 108 | 122 | 121 | 199 |
| T5 ♂ | 31.v.54 | — | 15 | 25 | 25 | — | — | — |
| T6 ♂ | 10.vi.54 | — | 34 | 985 | 104 | 112 | 112 | 223 |
| T7 ♂ | 17.vi.54 | — | 85 | 1058 | 106 | 113 | 121 | 190 |
| T8 ♀ | 19.vii.54 | — | 88 | 994 | 105 | 119 | 123 | 189 |
| T9 ♀ | 21.v.55 | — | 31 | 610 | 99 | 106 | 137 | 316 |
| T10 ♂ | 9.vi.55 | 1.5 ± 1.5 | — | 43.5 | 43.5 | — | — | — |
| T11 ♀ | 11.vii.55 | — | 119 | 158 | — | 119 | 120 | — |
| T12 ? | 11.viii.55 | 1.5 ± 1.5 | — | 1.5 | 1.5 | — | — | — |
| T13 ♀ | 12.viii.55 | — | 144 | 446 | — | 144 | ? | 192 |
| T14 ♀ | 12.viii.55 | — | 124 | 656 | — | 124 | 137 | 330 |
| T15 ♂ | 12.ix.55 | 2 ± 2 | — | 488 | 110 | 114 | 128 | 194 |
| T16 ♀ | 19.ix.55 | — | 145 | 641 | — | 145 | ? | 225 |
| T17 ♂ | 21.x.55 | — | 8 | 512 | 119 | 126 | 140 | 196 |
| T18 ♀ | 27.x.55 | 7 ± 7 | — | 448 | 119 | 126 | 133 | 217 |
| T19 ? | 27.x.55 | 7 ± 7 | — | 7 | 7 | — | — | — |
| T20 ♀ | 12.iv.56 | 3.5 ± 3.5 | — | 325.5 | 101.5 | 108.5 | 115.5 | 158.5 |
| T21 ♂ | 12.iv.56 | 3.5 ± 3.5 | — | 24.5 | 24.5 | — | — | — |
| T22 ♂ | 12.iv.56 | 3.5 ± 3.5 | — | 325.5 | 108.5 | 115.5 | 129.5 | 158.5 |
| T23 ♀ | 12.iv.56 | 3.5 ± 3.5 | — | 325.5 | 108.5 | 115.5 | 143.5 | 158.5 |
| T24 ♀ | 19.iv.56 | 3.5 ± 3.5 | — | 318.5 | 87.5 | 101.5 | 129.5 | 164.5 |
| T25 ♀ | 10.v.56 | 3.5 ± 3.5 | — | 297.5 | 108.5 | 115.5 | 143.5 | 185.5 |
| T26 ♂ | 17.v.56 | 3.5 ± 3.5 | — | 413.5 | 101.5 | 108.5 | 136.5 | 150.5 |
| T27 ♂ | 5.vii.56 | 3.5 ± 3.5 | — | 241.5 | 115.5 | 122.5 | 135.5 | ‡ |
| T28 ♂ | 27.ix.56 | 3.5 ± 3.5 | — | 157.5 | 101.5 | 108.5 | 122.5 | ‡ |
| T29 ♂ | 8.xi.56 | 7 ± 7 | — | 49 | 49 | — | — | — |
| T30 ♀ | 12.xii.56 | — | 83 | 83 | 83 | — | — | — |
| T32 ♀ | 16.v.57 | 4 ± 4 | — | 5 | 5 | — | — | — |

* Born in captivity. † Not born in captivity. ‡ Not separated.

TABLE II

*Trichosurus Vulpecula*

Dimensions of Specimens and Equations for Curves Shown in Figure 1

T4 ♂ — Born in the wild. For other details see Table I

| Estimated Age (days) | Head length (mm.) | Ear length (mm.) | Manus length (mm.) | Pes length (mm.) | Tail length (mm.) | Body weight (gm.) |
|---|---|---|---|---|---|---|
| 24 | 14.3 | — | — | 5.3 | 12.5 | — |
| 31 | 17.2 | 4.4 | — | 7.0 | 19.7 | — |
| 38 | 18.7 | 7.3 | — | 8.8 | 26.5 | — |
| 45 | 22.4 | 8.6 | — | 10.9 | 32.8 | — |
| 52 | 24.8 | — | — | 13.0 | 40.0 | — |
| 66 | 30.5 | 15.6 | — | 17.3 | 55.6 | — |
| 80 | 37.0 | — | — | 22.5 | 69.3 | — |
| 94 | 40.7 | 22.8 | — | 26.9 | 87.3 | — |
| 108 | 48.0 | 26.4 | 34.4 | 33.6 | 100.0 | — |
| 122 | 54.5 | 33.9 | 45.5 | 42.8 | 137.0 | 156 |
| 136 | 59.4 | 41.5 | 49.9 | 50.2 | 165.0 | 234 |
| 150 | 64.0 | 44.1 | 54.6 | 55.3 | 188.0 | 354 |
| 157 | 66.5 | 49.9 | 62.8 | 59.2 | 200.0 | 443 |
| 164 | 66.4 | 52.4 | 65.0 | 62.9 | 214.0 | 527 |
| 171 | 69.5 | 57.2 | 67.3 | 65.2 | 230.0 | 596 |
| 178 | 72.3 | 56.3 | 65.5 | 68.0 | 232.0 | 690 |
| 192 | 76.7 | 61.7 | 70.5 | 70.0 | 252.0 | 946 |
| 206 | 77.3 | 64.1 | 71.7 | 71.6 | 273.5 | 1167 |
| 227 | 81.8 | 64.9 | 74.3 | 74.6 | 275.0 | 1390 |
| 234 | 85.5 | 67.0 | 74.5 | 74.8 | 277.0 | 1515 |
| 255 | 83.9 | 66.5 | 74.8 | 75.5 | 298.0 | 1687 |
| 262 | 87.1 | 68.1 | 77.1 | 76.0 | 295.0 | 1711 |
| 290 | 90.3 | 72.0 | 77.7 | 77.6 | 296.0 | 1993 |
| 325 | 88.4 | 72.0 | 77.7 | 76.3 | 295.0 | 2107 |
| 346 | 93.5 | 72.8 | 76.1 | 78.5 | 302.0 | 2159 |
| 402 | 94.4 | 72.6 | 80.5 | 79.0 | 304.3 | 2292 |
| 458 | 100.2 | 75.7 | 79.5 | 78.5 | 315.0 | 2411 |
| 549 | 95.0 | 74.5 | 80.9 | 77.2 | 310.0 | 2712 |
| 654 | 100.5 | 73.5 | 79.0 | 77.9 | 310.0 | 2830 |
| 871 | 100.2 | 72.5 | 79.3 | 77.0 | 305.0 | 2467 |
| 892 | 99.5 | 74.0 | 80.1 | 78.2 | 305.0 | 2586 |

$y = 5.36 + 0.3617t + 0.000276t^2$

$y = -2.05 + 0.2106t + 0.000616t^2$

$y = 0.25 + 0.1843t + 0.001128t^2$

$y = 1.09 + 0.4570t + 0.004980t^2$

$y = 99.76 - 128.00e - 0.008539t$

$y = 73.73 - 304.67e - 0.01623t$

$y = 79.57 - 288.97e - 0.01726t$

$y = 77.81 - 537.86e - 0.02189t$

$y = 303.20 - 1661.71e - 0.01792t$

TABLE II (*cont.*)

T18 ♀ — Born in captivity. For other details see Table I

| Known Age (± 7 days) | Head length (mm.) | | Ear length (mm.) | | Manus length (mm.) | | Pes length (mm.) | | Tail length (mm.) | | Body weight (gm.) |
|---|---|---|---|---|---|---|---|---|---|---|---|
| 7 | 6.8 | $y = 3.71 + 0.3620t + 0.0002316t^2$ | — | $y = -2.10 + 0.1439t + 0.001211t^2$ | — | $y = 20.43 - 0.3216t + 0.003732t^2$ | — | $y = 2.18 + 0.0663t + 0.001738t^2$ | — | $y = 6.12 + 0.0951t + 0.0072551t^2$ | — |
| 21 | 11.0 | | — | | — | | 4.0 | | 10.3 | | — |
| 35 | 16.0 | | 4.2 | | — | | 7.0 | | 19.2 | | — |
| 70 | 31.0 | | 14.7 | | 16.2 | | 15.7 | | 50.0 | | — |
| 105 | 44.0 | | 25.0 | | 27.8 | | 27.2 | | 93.0 | | 66 |
| 119 | 48.7 | | 33.0 | | 35.0 | | 35.4 | | 120.8 | | 104 |
| 133 | 54.8 | $y = 88.44 - 132.02e - 0.01019t$ | 41.2 | $y = 68.57 - 268.30e - 0.01719t$ | 41.7 | $y = 66.40 - 208.50e - 0.01620t$ | 43.7 | $y = 72.27 - 179.78e - 0.01388t$ | 148.0 | $y = 294.29 - 710.17e - 0.01227t$ | 166 |
| 154 | 60.9 | | 49.4 | | 48.5 | | 52.3 | | 185.0 | | 313 |
| 189 | 69.5 | | 58.7 | | 57.3 | | 56.8 | | 225.0 | | 572 |
| 217 | 75.0 | | 63.0 | | 61.3 | | 65.3 | | 250.0 | | 826 |
| 329 | 83.2 | | 67.2 | | 64.7 | | 70.5 | | 280.0 | | 1507 |
| 378 | 86.4 | | 70.0 | | 66.2 | | 72.8 | | 290.0 | | 1562 |
| 399 | 86.8 | | 68.0 | | 66.0 | | 71.0 | | 292.0 | | 1693 |
| 448 | 88.0 | | 69.1 | | 68.0 | | 71.2 | | 294.0 | | 1911 |

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# THE INFLUENCE OF LIGHT ON THE EARLY GROWTH OF CHINOOK SALMON

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## INTRODUCTION

It is the objective of this study to increase the knowledge of the effects of the visible portion of the spectrum on salmonoids.

Under natural conditions, salmonoid eggs are deposited and develop under six to twenty-four inches of gravel. Throughout the length of the yolk-sac absorption stage, which varies according to the water temperature, the fry remain beneath the surface of the gravel. When the yolk-sac has been absorbed, the young salmon emerge from the gravel into the stream or lake, and commence feeding.

Under the conditions by which salmonoids are artificially propagated, the amount of light that the eggs, larvae, and fingerlings receive is highly variable.

## LITERATURE REVIEW

Smith (1), in his work on chinook salmon fingerlings, found that dark-reared fish weighed 41% more than light-reared fish after eight months of feeding. It was also noted that the dark-reared fish were much more uniform in size, and had a 2% lower mortality than the light-reared fish. Dannevig (2, 3) observed that cod fry, 4-8 days old, showed a positive phototaxis to sunlight and to artificial light while confined in experimental tanks. The wave-length range of the artificial light was between 4000 and 270 A. It was also noted that 40 cod raised in a salt-water pond 5-meters deep had a lower average vertebral count (49.48) than 250 cod from the nearby sea (51.12). Conditions were similar except for more light (shallower water), and higher temperatures, in the pond. Dannevig and Sivertsen (4) found that cod larvae are attracted by moderate light, but die when exposed

to strong light. McHugh (5) observed that the vertebral count of the grunion, *Leuresthes tenuis,* may be significantly influenced by light. It was found that there were fewer vertebrae present if more light was present during the first month of life than if the light was subdued or absent.

## Methods and Materials

The experimental animals used in this study were chinook salmon fingerlings (*Oncorhynchus tshawytscha*) of a common parentage. The fingerlings had been reared in darkness and had been actively feeding on raw, ground, beef-liver for one week.

For twelve weeks, four groups of 100 fingerlings each, were continuously subjected to intensities of 0.02, 88, 116, and 157 foot-candles, respectively, of a fluorescent light source characterized by a wave-length range of 3350-6000 A. (a range which excludes infra-red, and ultra-violet radiation) and a mean at approximately 5800 A. (yellow portion of the spectrum). The light from each tube was spectrographically analyzed before and after the experiment with no apparent change in either wave-length range, or mean. The intensities, as measured with a Photovolt foot-candle meter, remained constant throughout the length of the study.

Each group of fingerlings was confined in a different trough in a space measuring 44 inches long, 12 inches wide, and 5-6 inches deep. Perforated, stainless-steel plates comprised the ends of this space, with the bottoms and sides of cement that had been painted with a non-toxic aquamarine paint, and a clear coat of Tropalite waterproof paint. A water-flow of 1.8 gallons per minute, as well as a uniform temperature and water source, were maintained for each trough. A 1/16 inch thick plate of window glass was placed between the light source and the fingerlings in order to eliminate any long ultra-violet rays emitted. A one-layer thickness of ordinary window screen was similarly placed in order to shield the fingerlings from any radio-waves. All of the glass plates were cut from the same large strip, and all of the screen came from one roll.

Each group of fingerlings was fed a surfeit of ground, raw, beef-liver three times daily. Each trough was siphoned daily, and the uneaten liver and waste products removed.

At two-week intervals, fifty fingerlings from each group were randomly selected, and their lengths and weights recorded. The finger-

lings were impounded in a net, and all excess moisture permitted to drain for 15 seconds prior to being deposited in a counterbalanced pan of water. The weighing was done on an Ohaus beam balance, and the combined weights of the fingerlings were recorded to the nearest gram. After weighing, individual length measurements between the tip of the snout and the end of the scaled region were taken. This process was accomplished with the aid of a metal funnel fastened, with beeswax, to a curved glass tube. The fingerlings were measured through the glass tube with a pair of dividers, and this distance converted into a mm. measurement by use of a metric ruler.

## Treatment of Data

At the end of the study, a length-weight regression line was plotted for each group. The transformed formula used to plot the individual regression lines was:

$$\text{Log } W = \text{Log } A + B \text{ Log } L.$$

$W = Y =$ mean fingerling weight

$L = X =$ mean fingerling length

$A =$ constant: $\Sigma Y/n$

$$B = \text{slope} = \frac{\Sigma XY - \frac{(\Sigma X)\ (\Sigma Y)}{n}}{\Sigma X^2 - \frac{(\Sigma X)^2}{n}}$$

The standard analysis of covariance test was applied to these data in order to test the hypothesis that there was no difference among the adjusted means of these groups.

The mean length gains were analyzed by the "t" test statistic (6):

$$t = \frac{\overline{X} - \overline{Y}}{Sp \sqrt{\frac{1}{nx} + \frac{1}{ny}}}$$

$\overline{X}, \overline{Y} =$ sample means

$$Sp^2 = \frac{(nx - 1)\ (Sx^2) + (ny - 1)\ (Sy^2)}{nx + ny - 2}$$

$nx, ny =$ sample sizes

$Sx^2, Sy^2 =$ variances of the respective samples

Each group was tested against every other group regarding mean length gain as the random variable (presented in Table 2).

The weight gains could not be analyzed by the use of the "t" test statistic due to the lack of individual measurements (no variance); accordingly, analyses of covariance (with the time in weeks as the X variable, and the mean fingerling weight as the Y variable) for the period of 0-6 weeks, and 6-12 weeks were calculated.

A summary of the data are presented in Table 1.

## Results and Discussion

Results show that the light-reared groups are 40.5%, 41.8%, and 41.3% longer and 56.2%, 58.9%, and 56.9% heavier than the dark-reared group (Table 1). These results are not in agreement with those reported by Smith. Smith based his conclusions concerning the growth rate of fingerlings on specimens that had been exposed to light since fertilization; therefore, a strict comparison can not be made between the present study and Smith's study.

Table 2 indicates that the light-reared fish are not only longer than the dark-reared group, but that there is no significant difference among the light-reared groups. This implies that the lowest experimental intensity (88f.c.) exceeds any threshold value between "light" and "dark"; similarly, the highest experimental intensity (157 f.c.) was not high enough to produce results significantly different from that of the 88 f.c group.

When weight (Y) was plotted against time (X), no difference ($F = .068$) was found among the groups for the first six weeks. A significant difference ($F = 8.03^*$) in the weight-time relationship was evident during the last six weeks.

The results of these analyses may be interpreted in three ways: the fingerlings are resistant to light effects during the first six weeks; the fingerlings are affected by visible light during the last six weeks; or the effects of light are cumulative with the threshold at the sixth week.

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* Significant at the 5% level.

TABLE 1
Summary of Observations

| Time Weeks | | Intensity in foot-candles 0.02 | 88 | 116 | 157 |
|---|---|---|---|---|---|
| 0 | Length (mm.) | 35.36 | 35.36 | 35.36 | 35.36 |
| | S.D. length* | .74 | .74 | .74 | .74 |
| | Weight (gms.) | .69 | .69 | .69 | .69 |
| 2 | Length | 38.66 | 37.80 | 38.34 | 38.56 |
| | S.D. length | .89 | 1.03 | .85 | .68 |
| | Weight | .84 | .84 | .84 | .86 |
| | Mortality | 0 | 0 | 1 | 0 |
| 4 | Length | 43.32 | 42.72 | 42.20 | 42.68 |
| | S.D. length | 1.25 | 2.71 | .99 | 1.38 |
| | Weight | 1.22 | 1.08 | 1.08 | 1.16 |
| | Mortality | 0 | 1 | 0 | 0 |
| 6 | Length | 49.08 | 49.90 | 49.02 | 49.46 |
| | S.D. length | 1.78 | 3.19 | 1.81 | 1.50 |
| | Weight | 1.60 | 1.70 | 1.64 | 1.70 |
| | Mortality | 0 | 1 | 0 | 0 |
| 8 | Length | 53.58 | 57.44 | 56.48 | 57.00 |
| | S.D. length | 3.08 | 3.28 | 2.47 | 2.70 |
| | Weight | 2.34 | 2.64 | 2.54 | 2.72 |
| | Mortality | 1 | 0 | 1 | 1 |
| 10 | Length | 57.40 | 65.22 | 65.22 | 65.40 |
| | S.D. length | 3.61 | 3.85 | 2.78 | 3.64 |
| | Weight | 2.78 | 3.98 | 3.68 | 3.96 |
| | Mortality | 0 | 1 | 0 | 1 |
| 12 | Length | 62.74 | 73.84 | 74.18 | 74.06 |
| | S.D. length | 4.67 | 5.19 | 4.10 | 5.02 |
| | Weight | 3.68 | 5.36 | 5.44 | 5.38 |
| | Mortality | 0 | 1 | 0 | 0 |
| | Total % mortality | 1 | 4 | 2 | 2 |
| | % gain over dark-reared group | | | | |
| | in length | x | 40.5 | 41.8 | 41.3 |
| | in weight | x | 56.2 | 58.8 | 56.9 |

The water temperature rose consistently over the twelve-week period from 7.5°C. to 11.3°C.

* Standard deviation of the lengths.

TABLE 2
Analyses of Mean Length Gains by the *t* Test

| Group | Mean Length Gain, in mm. | Group 0.02 | 88 | 116 | 157 |
|---|---|---|---|---|---|
| 0.02 | 27.38 | x | 11.03** | 12.71** | 11.46** |
| 88 | 38.48 | x | x | 0.36 | 0.22 |
| 116 | 38.82 | x | x | x | 0.01 |
| 157 | 38.70 | x | x | x | x |

** Significant at the 1% level.

Due to the apparent increase in the metabolism of the light-reared fish, the last interpretation appears to be the most feasible. The increased metabolism manifested itself in the comparative activity of the light-reared groups when being measured, and the marked increase in activity at feeding time. That the growth of all groups was constant is indicated by the result of the length-weight analysis of covariance test ($F = 2.47$). The standard deviations of the lengths of each group were approximately the same (Table 1), implying that light or dark conditions did not cause any size disparity within each group.

The mortalities within groups ranged from 1% to 4% and could be directly attributed to mechanical injury sustained during the measuring processes; consequently, the differences are ignored.

## Summary and Conclusions

Four groups of chinook salmon fingerlings were fed a 100% beef-liver diet while continuously subjected to a light intensity of 0.02, 88, 116, and 157 foot-candles, respectively, of a light source characterized by a wave-length range of 3350-6000 A. and a mean at approximately 5800 A. No differences among groups regarding growth were apparent during the first six weeks of the twelve week study. By the end of the twelfth week, the light-reared groups weighed 56.2%, 58.9%, and 56.9% more, and were 40.5%, 41.8%, and 41.3% longer than the dark-reared group. Statistical tests indicate that these differences are not only significant, but that there is no difference among the light-reared groups regarding growth. The length-weight relationship was found to be the same for all groups.

It is concluded that, under the conditions of this experiment, high intensities of light emitted from white fluorescent tubes will act as a growth stimulator on actively feeding chinook salmon fingerlings.

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## BOOK REVIEW

*The Beginnings of Embryonic Development*. Edited by A. Tyler, R. C. von Borstel, and C. B. Metz. Publication No. 48 of the American Association for the Advancement of Science, viii + 400 pp., illus. Washington, D. C. 1957.

A symposium on the "Formation and Early Development of the Embryo," held at the 1955 Atlanta meeting of the AAAS, was the basis for this collection of papers. However, since only seven papers were presented, the field of coverage was expanded by the inclusion of six additional contributions, four of which are by European authors.

The thirteen papers represent a selected sample dealing with various problems of early development. They include both descriptive and experimental work on the biological and biochemical levels. The majority are review articles, only a few including new and original data.

The first paper presents original work by W. S. Vincent on the RNA of the nucleolus and cytoplasm of starfish oocytes. He suggests that the nucleolus may be the "duplicating machine with the gene providing the stencil . . . for the rapid synthesis of . . . specific molecules of RNA in large numbers . . . for transfer to the cytoplasm" (page 18). The second paper is a comprehensive review of the present status of the fertilizing theory by C. B. Metz. This approach was initiated by F. R. Lillie in 1911, and despite hundreds of papers written since that date, as Metz states (page 59), ". . . fertilization and especially the activation of the egg cannot as yet be described completely in terms of the interaction of known specific egg and sperm substances."

The third and fourth papers deal with aspects of mammalian gametes and fertilization. C. R. Austin and M. W. H. Bishop concentrate largely on the physiology of spermatozoa, whle M. C. Chang discusses the actual fertilization process and environment. The fifth paper is a review, by A. L. and L. H. Colwin, of the acrosome filament in relation to sperm entry in certain marine invertebrate eggs. It is profusely illustrated with sixteen cuts of text figures and photomicrographs, reproduced largely from their earlier work and that of J. C. Dan. The sixth paper is a report of the work of A. Monroy on

the differences between the protein extracts of fertilized and unfertilized sea urchin eggs. Some sort of internal molecular re-arrangement is suggested, although no changes in the molecular configuration of the proteins of the fraction after fertilization have been demonstrated thus far. The next paper, by R. C. von Borstel, deals with nucleocytoplasmic relations during the early development of Habrobracon. An interesting difference between the nucleus and cytoplasm of the egg of this form is described: photoreactivation can cause recovery of the ultraviolet irradiation damage to nuclei, but the cytoplasm is reported to constitute a non-photorecoverable system.

The eighth paper is a review by H. E. Lehman, describing methods and results of nuclear transplantation, both indirect and direct. The recent work of Briggs and King, using direct nuclear transplantation as a means of studying nuclear differentiation, is the outstanding contribution in this field. In the ninth paper, J. R. Gregg describes the morphogenesis and gives data on the metabolism of blocked (gastrula-arrested) hybrids between *Rana pipiens* females and *Rana sylvatica* males. The tenth paper, by J. R. Shaver, reviews the work on cytoplasmic particles in the early development of the sea urchin egg. The criteria for the identification of the various cytoplasmic particles (including mitochondria, microsomes, Golgi bodies, etc.), in living or fixed eggs or in homogenates, appear still to be unsettled. There is no evidence that any of these particles are directly concerned with the differentiation process.

The next paper deals with the experimental conditions, especially chemical, which affect early differentiation. It is a field in which the author, S. Ranzi, has made many contributions, using such agents as lithium and thiocyanate. The twelfth paper is by G. Reverberi, on the role of certain enzymes in ascidian development. The enzymes (and coenzymes) are supposed to be those associated with at least two different types of particles that various investigators have called "mitochondria." However, the present knowledge relating to localization of the enzymes is meager, and the experimental approach is to study the effects of enzyme inhibitors. These inhibitors produce general mesodermal or ectodermal abnormalities.

The last paper, by A. Tyler, deals with immunological studies of early development. The material presented includes a review of changes in antigenicity during embryogenesis, development of an-

tibody-forming capacity, and effects of antibodies on development. An account is given, also of the author's own experiments on the inhibition of cleavage in sea urchins with antisera prepared against fertilizin. No explanation is given as to why this agent should have any specific effect on the cleavage process.

Even though the book reflects some differences in outlook and in interpretation on the part of the various contributors, "these serve mainly to emphasize the lack of critical information concerning the particular problem under discussion" (p. iv). It will be useful in pointing out unsolved problems to new generations of students and investigators, in addition to constituting a measure of present progress in this important field.

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